The patterns of migration in anadromous three-spined stickleback (*Gasterosteus aculeatus*)

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Master of Research

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1. General introduction

1.1 Anadromous migration

Migration is a common occurrence in many organisms and environments, both terrestrial and aquatic. It is defined as the synchronised movement of part of or all of population of a species between two distinct habitats (Dingle & Drake, 2007). Migration occurs for many reasons: to avoid intraspecific and interspecific competition (Cox, 1968), to avoid predators (Brönmark, *et al.*, 2008), to be near conspecifics during breeding seasons (Wagner, *et al.*, 2009), and for better feeding grounds (Ounsley, *et al.*, 2020). Migration is a phenomenon that happens at least once in the life of most fishes. Fish migration occurs in a variety of spatial scales in both freshwater and seawater (Brönmark, *et al.*, 2013; Madigan, *et al*., 2014). Anadromous migration bridges the gap between freshwater and seawater with organisms migrating across a variety of salinity gradients (McDowall, 2001). Most often anadromous migration occurs in fishes that have spawning grounds in freshwater or saline lagoons (Forsythe, *et* al., 2012; McElroy, *et al.*, 2012; de Magalhães Lopes, *et al.*, 2018; Finlay, *et al.*, 2020). Little is known about why these anadromous migrations take place, but they are probably linked to the varying costs and benefits associated with the opposing ends of migratory routes. These are well documented in birds, which migrate towards the equator for warmer winters with more food and to avoid colder winters with less food, and in summer migrate to temperate or arctic areas for better food, fewer natural enemies and to avoid hot summers (Alerstam, 1993). In contrast, the timings and factors influencing anadromous migration of fishes is still poorly understood. Anadromous migration is relatively well studied in salmonids, it is known to be protandrous, dependent on environmental cues such as precipitation, lunar phase and water velocity, and also biotic factors such as predation (Morbey, 2000; McDowall, 2001; Crossin, *et al.*, 2004; Spence & Dick, 2014; Kelson & Carlson, 2019; Finlay, *et al.*, 2020; Ounsley, *et al.*, 2020). Anadromy in stickleback on the other hand has been given little attention.

Anadromous migration allows for an individual to reap the benefits that both freshwater and marine habitats. Spawning in freshwater and lagoons decreases predation risk upon eggs and juveniles, increasing survival rate (Schiewe, 2013). Whereas, living predominantly in marine habitats can yield faster and larger growth, by taking advantage of the more productive waters (Gross, *et al.*, 1988). However, anadromy does come with costs, including an increased mortality rate as well as higher energy expenditure (Jensen, *et al.*, 2019).

The timings of anadromous migration, although poorly understood in stickleback, has been studied in other species, and ideas for probable factors have developed. The phase of the moon is thought to have a strong effect on the timings of migration. Often the variation in illumination related to the full and new moons is associated with changes in the numbers of anadromous species caught (Yako, et al., 2002; Verhelst, et al., 2018), with higher illumination often having a negative effect on smaller individuals (Longland & Price, 1991; Yako*, et al.*, 2002; Forsythe, *et al.*, 2012; Sudo, *et al.*, 2014). The lunar phase is also linked with the spring and neap tides which causes fluctuations in the currents and water depths and is also thought to be an influencing factor in anadromous migration (Jonsson, et al., 2018). Other than the effects of the lunar cycle, other abiotic and biotic factors likely influence the migratory timings of anadromous fish. Precipitation is thought to correlate with the numbers of migrating anadromous fish (Yako, et al., 2002; Giri, et al., 2019). The timing of anadromous migration may also be related to the risk of predation associated with migrating at different periods of a breeding season (Brönmark, *et al.*, 2008). Protandry, which is the earlier migration of males, occurs in salmonids that migrate earlier in the breeding season to maximise reproductive success (Morbey, 2000; Finlay, *et al.*, 2020). In species that establish breeding territories, migration that is protandrous is thought to be driven by increasing reproductive success by establishing better territories earlier (Ketterson & Nolan, 1976). To date, most studies into anadromous migration have been conducted on species that are larger than stickleback, e.g. salmonids.

1.2 Three-spined stickleback – A model species

Three-spined stickleback (Gasterosteus aculeatus, hereafter "stickleback") are an exemplary species of common fish, spanning a wide geographical distribution across the northern hemisphere and living in a variety of habitats (Bell & Foster, 1994). Anadromous sticklebacks live most of their lives at sea and start to migrate inland to spawn in early spring (Bergström, *et al.*, 2015).

Stickleback, are a single species complex, divided into multiple divergent ecotypes that differ behaviourally and phenotypically (McKinnon & Rundle, 2002). Anadromous and freshwater pairs offer an interesting example of ecotypes that differ significantly in morphology, physiology and behaviour but share a common habitat at a certain period of their lives. Anadromous and freshwater/lagoon resident stickleback (Hereafter "residents") share breeding grounds but reproduce separately, rarely hybridizing (Dean et al., 2019).

Ecological speciation, in which the evolution of adaptive traits, and subsequent divergence into new species, is developed through ecologically dependent natural selection, is a longstanding explanation for the divergence of a species into new sub-species (Rundle and Nosil, 2005). The establishment of freshwater and lagoon resident stickleback is long thought to have originated from a shared ancestor with the anadromous stickleback following the adaptation of marine populations of stickleback to survive solely in freshwater and lagoon habitats following the last glacial period (Bell and Foster, 1994).

Phenotypic differences are one way to discriminate between resident and anadromous stickleback. Anadromous stickleback are normally larger than residents (Higuchi, et al., 1996) and, within ecotypes, females are usually larger than males (Aguirre, et al., 2008). Additionally, residents usually have fewer lateral bony plates than anadromous stickleback (Reimchen, 1983; Bolotovskiy, et al., 2018; Dean, et al., 2019). These plates have developed as an antipredator trait (Bell, 2001). The ecology of resident stickleback in freshwater is well understood in comparison stickleback in saltwater, especially the saltwater resident, which is an unusual ecotype.

1.3 This study

Studies were conducted on North Uist, Outer Hebrides, spanning 6 weeks in May and June 2021, during the breeding season of stickleback. Marine stickleback have colonised many lochs and lagoons across North Uist since the covering icesheet melted 16,000 years ago (Ballantyne, 2010). These colonisers have since developed different morphological characteristics in differing lochs and lagoons, making the island an area of ecological significance. To examine the possibility of site fidelity and if there are any spatial differences in migration, two saline lagoons on opposing sides of the island were chosen for monitoring the numbers of anadromous stickleback over time.

In this study we monitored the timings of migration in anadromous stickleback. To better understand the factors influencing this migration, we looked at evidence that relate to the possible costs and benefits of migration. Such evidence included body size (as a reflection of growth rates), parasites and marks of predation. Furthermore, this evidence might permit additional understanding of anadromous stickleback including site fidelity and protandry. In addition to the biotic factors, it is important to look at all possibilities of migration when little is already known. Therefore, we monitored lunar phase, water depth, precipitation as well as salinity which could be important in being able to piece together an idea of the factors influencing migration.

Secondly, we looked at the possibilities of ROVs being a suitable tool for estimating population densities of stickleback. To be able to determine the timings and factors influencing migration of anadromous stickleback, it is important to estimate population densities over an expected migration period. To date, estimating population densities in small teleost fish has often required the use of minnow traps, which may be subject to bias (Fernö & Olsen, 1994; Kressler, *et al*., 2021). With advancing technologies in remotely operated underwater vehicles (ROVs) the possibility of being able to use these drones for surveying population of stickleback has increased significantly. In recent decades ROVs have become more accessible to researchers with declining costs, personal ROVs can now cost ten times less than the cheapest drone from

2008 (Sward, *et al.*, 2019). Aerial drones have long been established as useful tools in terrestrial monitoring of wildlife, as a more accurate and cost effected method of surveying (Broich, et al., 2011; Christie, *et al.*, 2016). By comparing the catch per unit effort (CPUE) of minnow traps against observations of stickleback made by an ROV, we can analyse the feasibility of being able to use drone technology for conducting surveys of populations in stickleback. Additionally, analysis of the results of each method can present a better understanding of the differences in stickleback behaviour between ecotypes as well as distribution in a particular area.

2. Chapter 1

The patterns of migration in anadromous three-spined stickleback (*Gasterosteus aculeatus*)

Abstract

Studies on anadromous stickleback migration are limited, and its influencing factors near unknown. To establish the timings of anadromous migration in stickleback on North Uist, for 6 weeks we observed anadromous stickleback migrating through channels as well as resident stickleback at two sites on opposite sides of the island. By recording ecotype and sex, environmental factors of lunar phase, water depth and salinity, and taking measurements of standard length, marks of predation, and abundance of blackspot, we were able to better understand the costs and benefits of migration timing, and to begin to understand the factors that influence it. Our results showed an obvious sex variation in the timing of migration, with early migration being protandrous with smaller males migrated earlier in the breeding season, likely to establish advantageous territories to maximise reproductive success. Temporal effects on female migration appeared to be less based on reproductive success than on energy conservation: females arrived in two distinct pulses associated strongly with lunar phase. Migrating at periods of reduced energy expenditure allows for faster migration to minimise predation and reduces the need to feed to replenish energy for egg production.

2.1 Introduction

Migration occurs in most fishes at certain stages of their life, be it for food or for reproduction. Anadromous fishes live most of their lives at sea and come into inland water bodies of lower salinity to mate and spawn. Some of these migrations are spectacular and well studied (Quinn & Myers, 2004), while others go on with little human attention. Little is known about why these anadromous migrations take place, but they are probably linked to the varying costs and benefits associated with the opposing ends of migratory routes (Alves, *et al.*, 2013). These costs and benefits are well documented in birds, which migrate towards the equator for warmer winters with more food and to avoid colder winters with less food, and in summer migrate to temperate or arctic areas for better food, fewer natural enemies and to avoid hot summers (Lincoln & Peterson, 1979). In contrast, the anadromous migration of fishes is poorly understood including the timings and factors influencing it.

Previous studies into anadromy, have shown that there are a few probable abiotic factors that influence the timings of migration. Firstly, migration for anadromous fish is thought to be affected by lunar phase, with the new moon and full moon often seeing an increase in numbers migrating (Yako, *et al.*, 2002; Verhelst, *et al.*, 2018). Additionally, fluctuations in water depth have also been shown to likely influence migration (Jonsson, *et al.*, 2018). Rainfall volume also correlates with the numbers of migrating anadromous fish (Yako, *et al.*, 2002; Giri, *et al.*, 2019). Other to the environmental factors that influence migration, behavioural traits of a species also influence timings of migration. To maximise reproductive success, males often arrive at breeding grounds earlier than females, known as protandry. It has often been evidenced to occur within salmonids migrating into spawning grounds (Morbey, 2000; Finlay, *et al.*, 2020). This allows males to select ideal areas as territory and in some cases build nests. Although anadromous migration has been studied comparatively well in larger salmonids, anadromy is still an area of migration that is poorly understood and documented. Moreover, research into

anadromous migration in stickleback is limited, and the mechanisms and timing of migration are relatively unknown.

Three-spined stickleback (*Gasterosteus aculeatus*) are a very common fish with a wide geographical distribution in the northern hemisphere (Bell & Foster, 1994). An anadromous ecotype spends the greater portion of its life in the sea and only comes into lakes and rivers to breed. The anadromy of stickleback has led to the establishment of many freshwater and lagoon resident populations. Residents are believed to have originated from an ancestor shared with the anadromous stickleback since the last glacial period, when marine populations of stickleback adapted to survive solely in freshwater and lagoon habitats (Bell and Foster, 1994; Fang, *et al.*, 2020). Anadromous and resident three-spined stickleback are one of six genetically divergent distinct pairs of ecotypes of stickleback (McKinnon & Rundle, 2002). Developments in genomics have allowed for better understanding of the evolutionary history of sticklebacks in recent years. Marine and freshwater stickleback are shown to often have a shared origin for many loci with strongly differentiated allies (Jones, *et al.*, 2012). High levels of standing genetic variation in alleles can allow for rapid responses to changes in environment, facilitating parallel adaptive radiation in stickleback populations (Colosimo, *et al.*, 2005). Sticklebacks provide a useful example for understanding the systems that drive species divergence, a usefulness which is amplified when the divergent forms share a common location. Anadromous and resident stickleback share breeding location, and although breeding often occurs simultaneously, the ecotypes are mostly reproductively isolated, only hybridizing at a rate of ~1% (Dean *et al.,* 2019). Ecological speciation, in which the evolution of adaptive traits is developed through ecologically dependent natural selection, is a longstanding explanation for the divergence of a species into new sub-species (Rundle and Nosil, 2005). It is possible that factors relating to migration influence the reproductive isolation between these two ecotypes. The resident stickleback ecotype has been well studied, but migration in the anadromous ecotype, although being a significant part in stickleback life history is an area that has had little research and is still poorly understood.

Phenotypic differences are one way to discriminate between resident and anadromous stickleback. Resident and anadromous stickleback differ greatly on size, with the latter being the larger of the two ecotypes (Higuchi, *et al.*, 1996). In temperate climates, migration benefits stickleback as the oceans are more productive than freshwater allowing for larger and faster growth, although increased productivity allows for more predators, meaning that remaining in freshwater habitats reduces predation risk (Gross, *et al.*, 1988). In addition to size differences between ecotypes, sexual dimorphism is a source of variation in size, with females being larger than males within ecotypes (Aguirre, *et al.,* 2008). Another way that anadromous and freshwater resident three-spined stickleback differ morphologically is in the presence of bony lateral plates. Armoured lateral bony plates have evolved as an antipredator trait amongst stickleback (Bell, 2001). The number of lateral plates differs between resident and anadromous stickleback. Residents have significantly fewer lateral bony armour plates at an average of 8 or less, when compared to the average of anadromous stickleback with 30 or more plates (Reimchen, 1983; Bolotovskiy, *et al.,* 2018; Dean, *et al.,* 2019). Both freshwater and anadromous stickleback are known to breed between ages 1 or 2 years, although on North Uist it's primarily at 2 years (Karve, *et al.*, 2013, Singkam & MacColl, 2019).

To gain understanding of migration in anadromous stickleback it can benefit to look a variety of phenotypic traits between populations of differing locations, including between ecotypes. Because the general patterns of migration are poorly understood, it helps to look at factors that relate to the possible costs and benefits of migration. Such factors include body size, breeding condition, parasites and marks of predation. Furthermore, such factors allow for additional understanding of anadromous stickleback including site fidelity and protandry. Understanding migration patterns can provide framework to better comprehend speciation that occurs between stickleback that often coexist and breed within a shared location. This study aims to display the patterns observed in the anadromous migration, and attempts to understand whether

sex-based opportunity, changes in water depth and lunar phase, or salinity has an effect in driving these patterns.

2.1.1 Study area

Freshwater lochs and tidal lagoons cover almost one third of the area of North Uist (Ballantyne, 2010). In almost all the lochs and lagoons across North Uist, stickleback can be observed, with populations displaying significant variation in morphology. The lochs on North Uist are of particular interest as they show a wide variety of phenotypic traits in the different lochs over a small spatial area (Magalhaes, *et al.*, 2016). The main trait varieties are predominantly variations in body size, spines and armour. Coexistence of partially divergent three-spined stickleback is observed in most of the tidal lochs across North Uist (Dean et al 2019). The two distinct ecotypes in tidal lochs are known as lagoon resident and anadromous, differing behaviourally, phenotypically, and genetically (Dean et al 2019; Dean et al 2021).

2.1.2 Aims

In this study, we investigate the patterns of migration in populations of anadromous three-spined stickleback during a period of 6 weeks in two tidal lagoons on North Uist. We show that the patterns of migration in anadromous stickleback differs between sexes. Standard length (SL), breeding condition, blackspot abundance (encysted digenean trematode larva of unknown species) and marks of avian predation were measured and recorded during the period of monitoring. This study specifically addressed the following questions: What are the patterns in migration for anadromous stickleback? To what extent are these patterns a shared trait between sites? Are there any morphological factors that differ between sites? Can water depth, lunar phase and salinity be linked to any sex or location based migratory variations for anadromous stickleback, or variations between ecotypes? The results of this study not only support findings of previous studies, but also provide new insight into the timings and patterns of migration of anadromous stickleback.

2.2 Methods

Data from a 6-week monitoring survey from a primary and secondary site were compiled and used to describe trends in migration, size and condition of three-spined stickleback. Between 10th May to 19th June 2021 traps were set and collected on a regular basis from two sites in North Uist, Clachan-na-Luib (hereafter 'Clachan', 57°33'10.9"N 7°20'03.7"W), on the western side of North Uist was the primary site of monitoring, with Loch an Duin (hereafter 'Duin', 57°38'32.2"N 7°12'30.0"W) on the North-Eastern side being the secondary site (Fig. 1). The two sites were chosen because of both having narrow channels between the lochs and the sea. The channel at Clachan was approximately 20m wide and is fed through three inlet culverts under a road bridge. Duin has two separate channels into the sea, although only one was used for monitoring. Similar to Clachan, Duin is connected to the sea by a single pipe (larger than at Clachan) at both channels. The monitoring site at Duin became isolated at low tides (during spring tides), leaving a shallow pool in the connecting channel between the monitoring site and the inlet pipe. The monitoring site itself was located within a tidal lagoon and never dries.
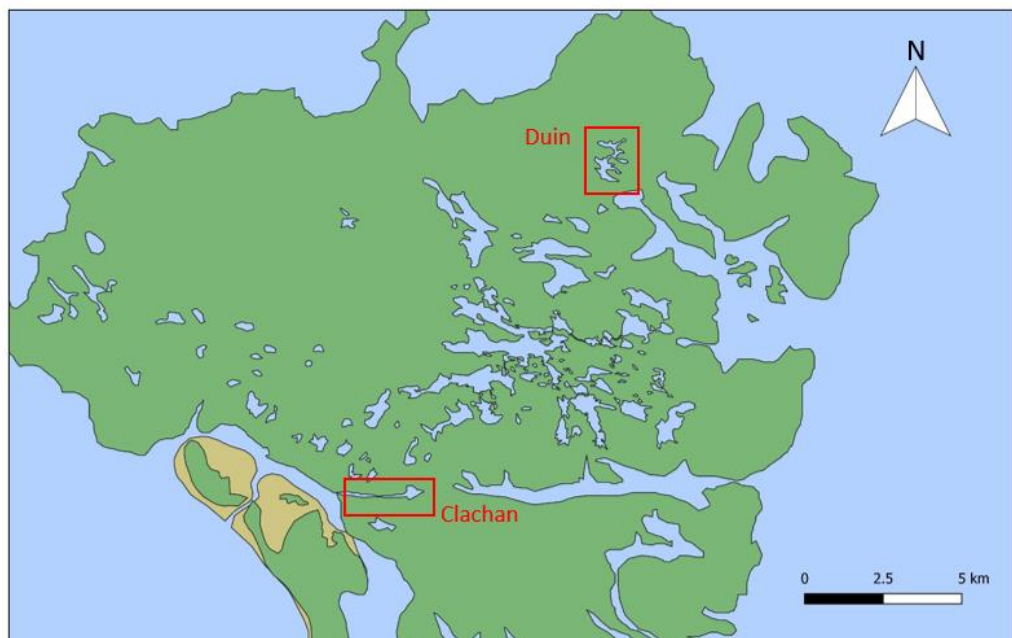
Figure 1. A map of North Uist showing the locations of the two lochs used for sampling anadromous and resident stickleback.

The area of channel used for the primary research site at Clachan, was shallower (~0.5 m) than the area used at Duin (~1.5 m). Duin connects to Loch nam Madadh before it reaches the sea in the east approximately 7km away. The depth of the water to the east of Loch nam Madadh decreases greatly from between 15-30m in the bay area to 60m+ (Marine Chart: GB_GB52770A, 2022). Clachan drains to the sea across tidal flats, approximately 5 Km to the open sea. By 8km west the water depth drops to an average of <10m. Up until approximately 25km west from Clachan the water depth reaches a maximum average of 30m.

2.2.1 Fish collection and sampling

Using unbaited minnow traps, sympatric resident loch and anadromous three-spined stickleback were caught, sampled and released *in situ*. Traps at Clachan were set and collected three times a week (Traps were not set on Sundays in respect of local religious customs) for a 6-week period. Duin was surveyed once per week for 5 weeks. 10 traps were set in pairs at 10m apart along a 50m stretch along the shore of the neck of the lochs. Traps were set for a 24-hour period and then retrieved the following day at approximately 18:00. Each pair at Clachan consisted of one new and one old trap, traps were cast and retrieved randomly to negate any bias.

Every stickleback caught was counted, and sex and ecotype were determined and noted. Ecotype was determined by a combination of size and presence of lateral plates, with a larger size and more plates being expected for the anadromous ecotype. For each collection day the first 5 stickleback of differing ecotype and sex (up to 20 total fish per day) were sampled for standard length, breeding condition and black spot. Standard length was measured from the anterior most point to the posterior edge of the caudal peduncle. Breeding condition was recorded as either breeding or not and was determined through visible nuptial colouration in males and females being noticeably gravid. A stickleback was given a yes/no for signs of blackspot. Any stickleback of undetermined sex was noted as being of unknown sex and were not used for

measurements. Marks of predation were also recorded from the 1st June on all observed stickleback. These marks were distinct 'V' shape scars from dorsal to ventral ("aviscars" (Reimchen, 1988), Fig. 2.), caused by the fish being seized and manipulated from above by avian piscivores (likely mainly terns, *Sterna* spp.).
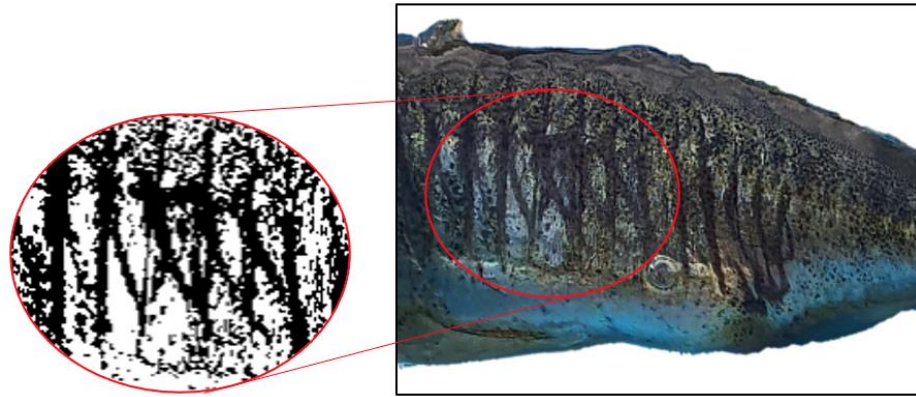
Figure 2. Example of aviscars on the side of a female anadromous stickleback.

Additionally, environmental variables were recorded at the trapping sites, including water depth, lunar phase, salinity and daily rainfall. Water depth was measured using a weighted rope and measuring tape, at the same time daily from the same location from the bridge over Clachan. Data on water depth were collected on both casting days and collection days. Salinity was recorded at both Clachan and Duin using a refractometer and samples of water taken from each of the trapping locations (Table 1, Fig. 1, Fig. 2). For Clachan salinity was recorded at high tide and low tide and additional salinity readings were taken from three extra locations for saltwater and loch water. Precipitation data were recorded through the Scottish Environmental Protection Agency (SEPA) monitoring station in Benbecula. Lunar phase was recorded daily using *timeanddate* and its data available for lunar phase for the Outer Hebrides (https://www.timeanddate.com/moon/phases/uk/stornoway, 2022).

Figure 3. A map of Clachan-na-Luib, red circles show the locations of the trap points and collection of salinity measurements.

Figure 4. A map of Loch an Duin and the locations of the five stickleback sampling locations (Red circles).

Table 1. Coordinates of the points of collection at Clachan-na-Luib and Loch an Duin. For Clachan-na-Luib. A, B, C, D, E are the trap points in ascending distance from the bridge respectively. Salinity was additionally measured at position S and L_1 for sea water and loch water respectively.

Loch	Position	N	W
Clachan	S	57°33'17.0	007°20'11.5
	A	57°33'16.8	007°20'00.4
	B	57°33'16.8	007°19'99.4
	C	57°33'16.8	007°19'98.4
	D	57°33'16.7	007°19'97.5
	E	57°33'16.6	007°19'96.4
	L_1	57°33'22.3	007°19'35.7
	L_2	57°33'18.0	007°18'59.0
Duin	A	57°38'32.2	007°12'29.4
	B	57°38'32.4	007°12'30.1
	C	57°38'32.5	007°12'30.6
	D	57°38'32.7	007°12'31.2
	E	57°38'32.9	007°12'31.7

2.2.2 Data analysis

Data were compiled in Excel (v.16.54), then analysis was performed in R (v.1.4.1717). A linear regression model was used to determine the correlation between standard length and time, sex and location, as well as for ecotypes and salinity.

A Chi-squared tests were used for comparisons of salinity, blackspot, breeding, predation and ecotype between sites and within sites. Following this a linear regression analysis was used again to investigate whether there was evidence of a relationship between standard length and date at Clachan, that differed between the sexes. This was not repeated at Duin because of insufficient temporal resolution of data. A Pearson's Chi-squared test was used to test

differences in sex ratios of anadromous stickleback between locations, as well as comparisons of ecotype breeding between sites. Additionally, a Welch two sample t-test was used for both sexes of anadromous stickleback at clachan to compare numbers caught in the first half to second half of the trapping period. A Fisher's exact test was used for blackspot, sex and location. A Fisher's exact test was also used for comparison of predation and location. A generalised linear model was used to compare number of females caught at Clachan against both depth and lunar phase as a modulus of cosine with corresponding values -2,-1,0,1,2 days from the catch date. A generalised additive model was used for comparing numbers of both ecotypes caught and salinity. Manipulation of dates was used for both comparisons of water depth to lunar phase, and numbers of anadromous females to water depth to determine whether there was a better correlation between two factors on different dates rather than the same date. For comparisons using lunar phase as water depth, data was often transformed using the modulus of cosine. This was select as the modulus of cosine best represents how the tides follow the lunar cycle.

2.3 Results

2.3.1 Overview of sites

In all, 1014 sticklebacks were caught over a 6-week period between 10th May and 19th June at the lochs Clachan and Duin. Of these, 280 were sampled for standard length, condition, blackspot and marks of predation. For each site, a maximum of 5 stickleback of the same sex and ecotype were sampled on any given collection day (totalling a maximum of 20 stickleback per day). The total number of trap collection days for Clachan was 17 and 5 days for Duin.

Table 2. The salinity (ppt) measured at both sites and all trap positions, including sea water (S) and loch water (L).

Location	Clachan (High tide) (ppt)	Clachan (Low tide) (ppt)	Duin (High tide) (ppt)
S	34	33	N/A
A	32	32	23
B	31	32	22
C	29	28	21
D	29	30	21
E	29	27	20
L_1	23	23	N/A
L_2	N/A	23	N/A

Both sites show a variation in salinity of 3ppt at high tide over the 50m area used for trapping (Table 2.). The salinity of Clachan was approximately 8-9ppt higher than that of Duin for each corresponding trap location. There is little variation in salinity at Clachan with regards to tidal height, with the salinity varying a maximum of 2ppt. Salinity measurements for Clachan were also taken from three additional locations (Sea water (S) and two loch waters (L_1+L_2)). The salinity of position S is approximately the salinity associated with sea water (35ppt). The consistency of salinity of loch water (L_1=23ppt, L_2=23ppt) at Clachan suggests that the loch reaches a point of equilibrium from L_1. If salinity declines at a constant rate at Clachan, it would be expected that it would reach the salinity of the main lagoon (23ppt) within a further 30-60m. The highest measured salinity of Duin was recorded at 23ppt, equal to the lowest recorded salinity of Clachan. The distribution of anadromous and resident stickleback was significantly affected by salinity (Fig. 5., $\chi^2 = 2787.4$, p-value = 0.00049, Pearson's Chi-squared test with simulated p-values). Distribution of anadromous stickleback does not to differ over varying salinity (Fig. 5., Estimate = 2.6957, Std. Error = 0.2201, t-value = 12.25, smooth terms: edf=5, p-value =0.13, R^2=0.0156, generalised additive model), whereas number of residents declines from areas of low to high salinity (Fig.2.,

Estimate =1.9652, Std. Error = 0.2015, t-value = 9.754, smooth terms: edf=5, p-value =<2e-16, R^2=0.53, generalised additive model).
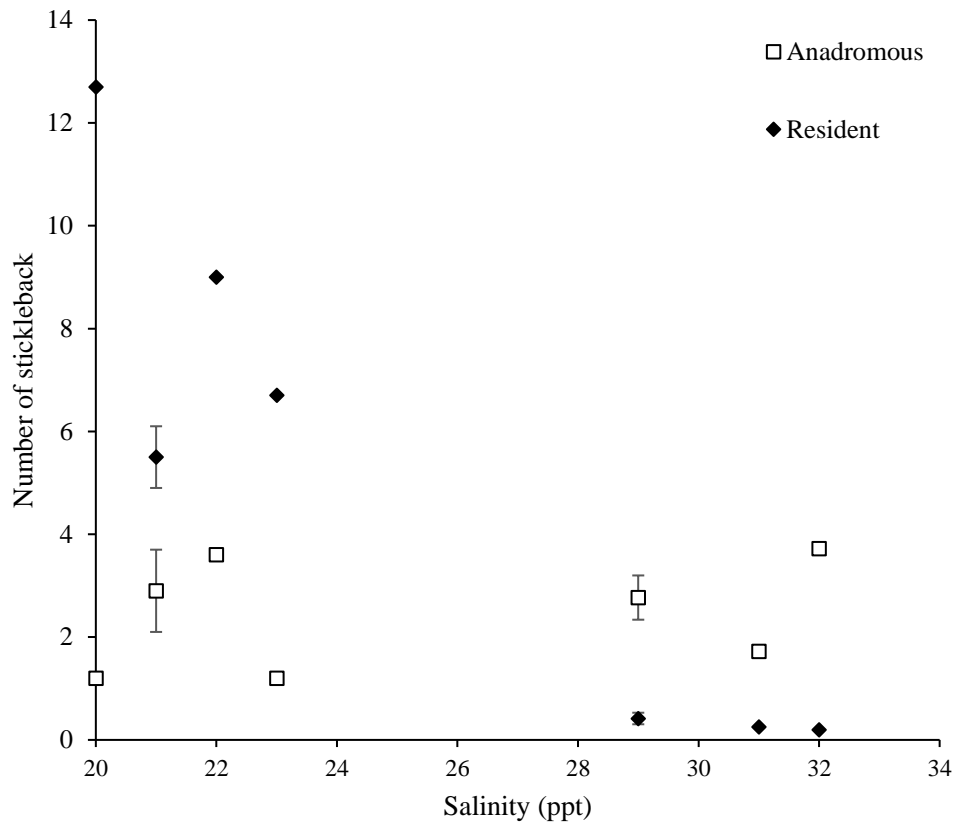
Figure 5. The average number of stickleback caught per trap of each ecotype with varying salinity from both Clachan and Duin (Salinity data from high tides).
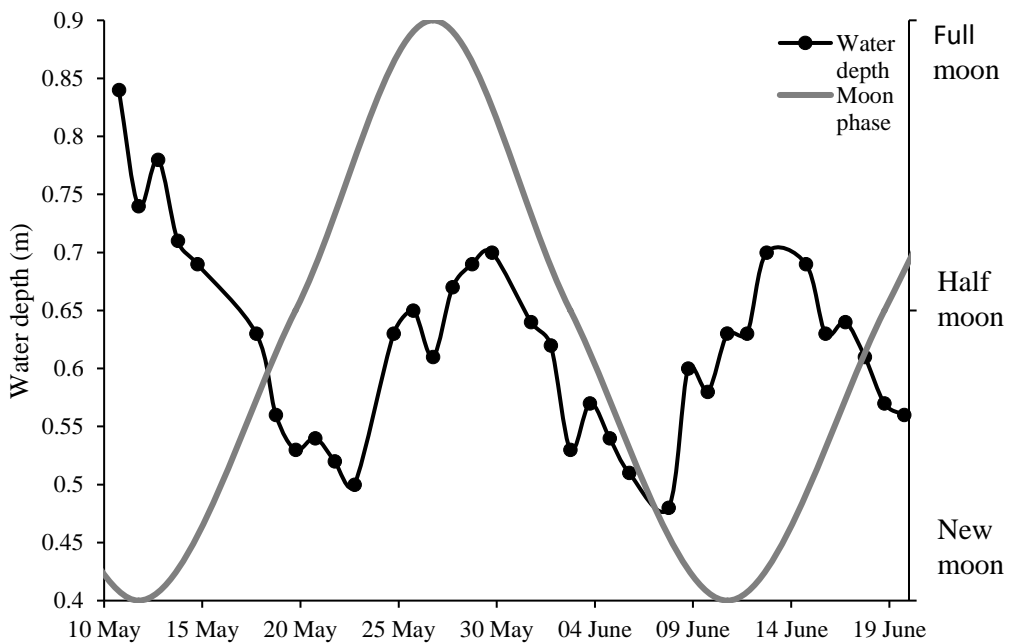
Figure 6. The relationship between measured water depth (m) and lunar phase measured at the Clachan bridge at the same time on each setting or lifting day.

At Clachan, water depth follows the same trend as the lunar phase, following tidal expectations (Fig. 6.), with the depth of water peaking at approximately 0.7m two days after both the new and full moon (29th May and 12th June). The higher water depth before 13th May 2021 can likely be linked to the 68.4mm of rainfall measured between the 1st and 12th May (SEPA). The following days of May only saw a total of 32.2 mm of rainfall, resulting in more consistent levels of measured water depth. Maximum water depth occurs after a 2 to 3 day delay from the new and full moons. This trend is evidence more when comparing the water depth against lunar phase as a modulus of cosine (Fig. 7.). This time lag is likely the result of the distance of the loch from the sea.
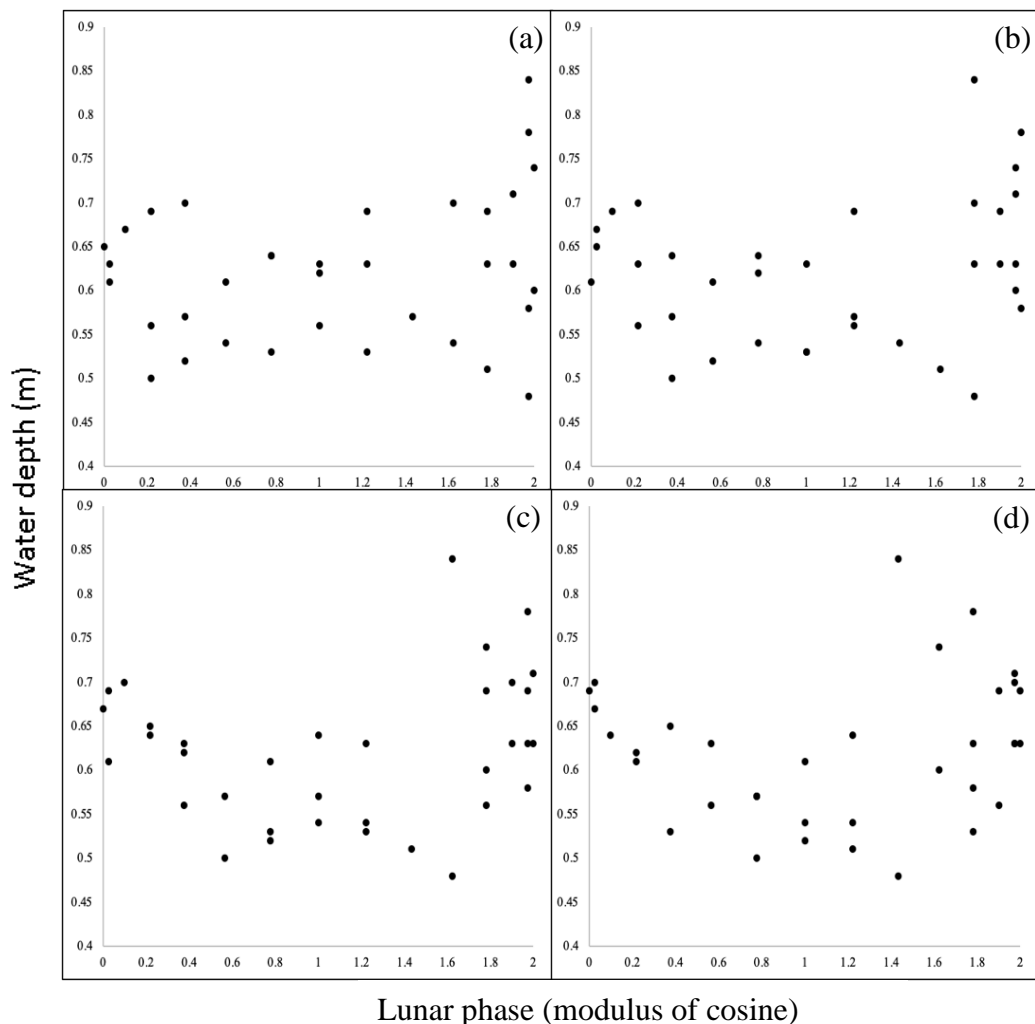
Figure 7. The relationship between depth and corresponding lunar phase (0=full moon 2= new moon); (a) on the same day, (b) 1 day before, (c) 2 days before, and (d) 3 days before. All values for lunar phase use modulus of cosine.
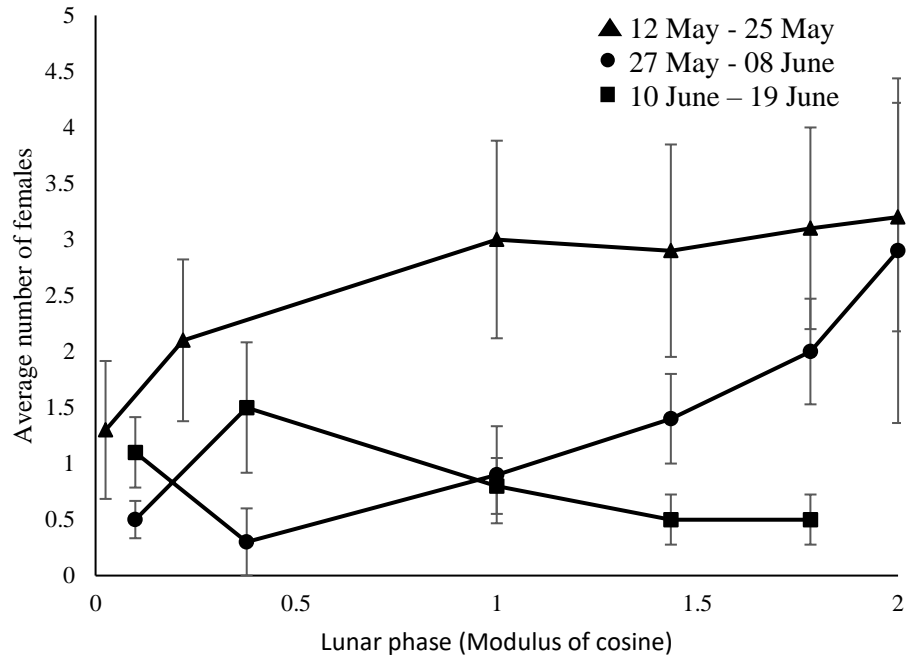
Figure 8. Average number of female stickleback caught per trap against the lunar phase (modulus cosine wave for after full moon, modulus cosine wave*-1 for after new moon), 0= full and new moon, 1=half-moons. Error bars = standard error.

The greatest number of anadromous females occurs be just before the full moon (Fig. 8.). The days before a spring tide saw a high number of female anadromous stickleback caught (Range [n] 20 – 32). The days after spring tide saw a lower number of females (Range [n] 3-15). The number of anadromous females increases from the new and full moons over a 14-day cycle, before dropping sharply at the following corresponding new or full moon. Although there was a general trend of increasing catch rate from the new and full moons, there was no significant variation between the numbers caught in the first week after to the second week after a full/new moon (Std.Error = 0.016, t-value = 2.089, p-value = 0.054, R^2 = 0.225, Generalised linear model). The final trapping period after the second new moon decreased in number caught rather than increased like the first two trapping periods (Fig. 8.).

Table 3. Summary of stickleback caught at Clachan-na-Luib. The total number of sticklebacks observed and number of those sampled for standard length and breeding condition (coloured-up or visually gravid).

Ecotype and sex	Total number	Sampled number	Standard length (mm)	Range (mm)	Number of breeding stickleback	Total breeding stickleback in sampled population (%)
Anadromous female	292	83	66.97	55 - 79.4	31	37.3
Anadromous male	115	57	63.66	53.6 - 72.45	15	26.3
Anadromous unknown	37	0	n/a	n/a	n/a	n/a
Resident female	49	41	39.78	31.6 - 48.4	23	56.1
Resident male	8	8	37.57	34.05 - 41.75	3	42.9
Resident unknown	1	0	n/a	n/a	n/a	n/a

2.3.2 Anadromous and resident

Stickleback of two ecotypes (Anadromous and Resident) were caught at both Clachan and Duin. Overall, of the stickleback caught over the 6 weeks at Clachan 88% were anadromous (Table 3.).

Males of both ecotypes were caught at a much lower rate than females (14% of total residents were male and 26% of total anadromous were male). Almost three times more anadromous females (n=292) were caught than males (n=115) in the channel at Clachan (Table 3., $\chi^2 = 76.975$, df = 1, p-value $< 2.2e-16$, Pearson's Chi-squared test with Yates' continuity correction). Similarly, more resident females (n=49) than males (n=8) were recorded.
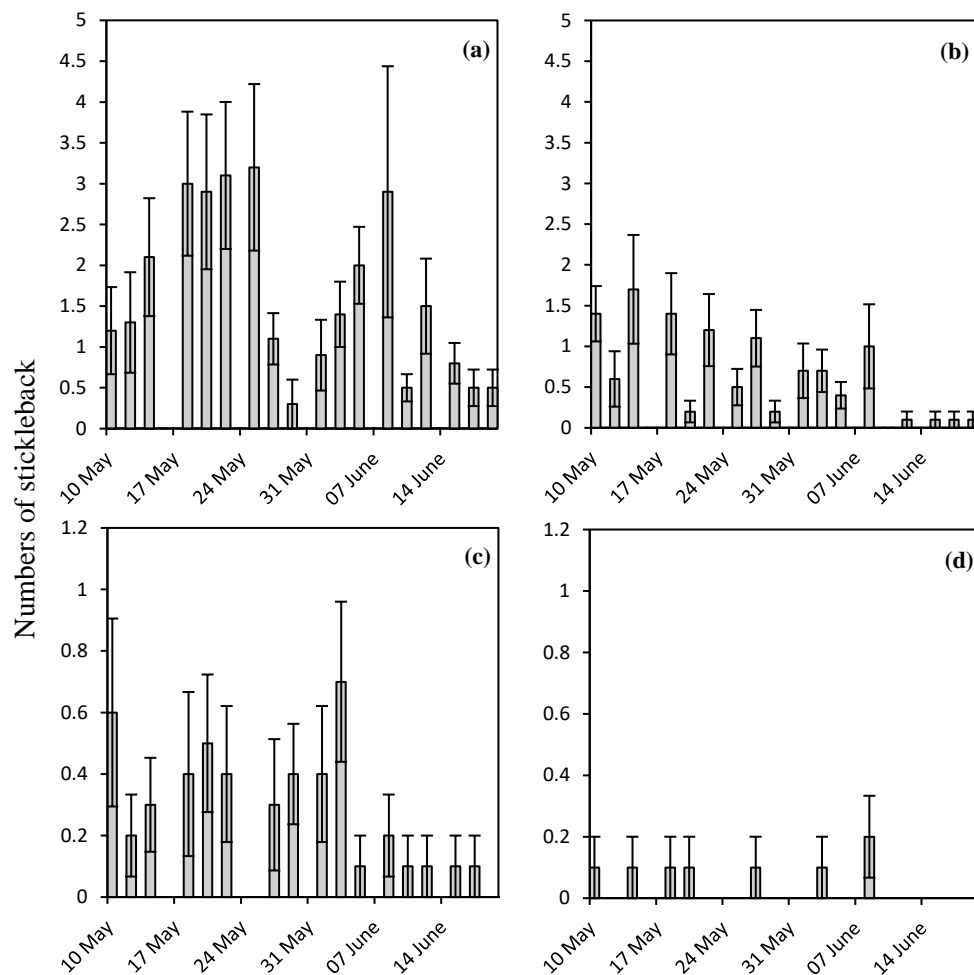
Figure 9. Average number of sticklebacks of both ecotypes and sex caught per trap between 10th May and 19th June 2021 at Clachan. (a) Female anadromous, (b) Male anadromous, (c) Female resident and (d) Male resident. Error bars = Standard error.

There were two peaks in numbers of female anadromous caught, the 25th May and 8th June (Fig. 9a), both of which occurred <2 days before the full and new moon respectively (Fig. 8). Of the two "pulses" in numbers of female anadromous stickleback, the first pulse had more caught than the second (182 in first 3 weeks and 101 in second 3 weeks) although did not differ significantly (t-value = 1.772, df. = 14.934, p-value = 0.097, Welch two sample t-test). With the consideration that unknown anadromous stickleback might be females, the first pulse becomes even greater in numbers. The two pulse trend is not seen with anadromous males (Fig. 9b). The highest number of anadromous males was caught towards the start of the 6 week period (17th May, n=17) and declined over time. When unknown anadromous stickleback are taken into consideration, it increases the trend in the decline of numbers over time. Significantly more anadromous males were caught in the first 3 weeks opposed to the following 3 (t-value = 2.556, df. = 13.69, p-value = 0.023, Welch two sample t-test).

Resident female numbers stayed rather consistent over the first 4 weeks and reduce to an average of approximately 1 caught per day (Fig. 9c) after 5th June Comparably, the same trend of reduction in numbers in the last 2 weeks and especially in the final week of trapping can be seen for all other ecotypes. Male resident stickleback were caught in the lower numbers, with between 0-2 being caught per week for all traps (Fig. 9d).
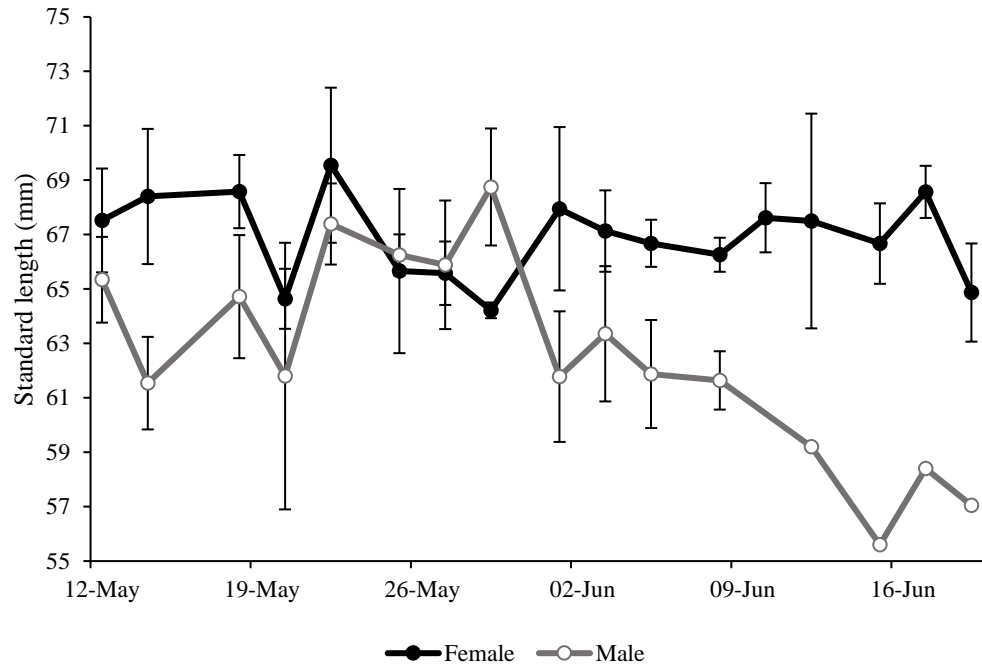
Figure 10. The mean (±SE) standard length of anadromous stickleback caught at Clachan-na-Luib, May to June 2021.

On average the standard length of female anadromous stickleback was longer than in males (Table 3., Estimate = -1.142e+01, Std. Error = 5.351e+00, t-value = -2.134, p-value =0.0347, linear regression analysis). The standard length of anadromous females remained relatively consistent over the 6 weeks observed (Fig. 10, Estimate = -0.02036, Std-error = 0.04045, t-value = -0.503, p-value = 0.616, linear regression analysis). In contrast, males appeared to decrease in standard length by the end of the trapping period. However, inspection of the data suggested that there was an initial increase in the length of males, and this was supported by a posthoc analysis which showed that the data for males were better fitted by a quadratic (Estimate = -0.014026, Std. Error = 0.005066, t-value = 2.769, p-value =0.0077, linear regression analysis), than a linear relationship (Estimate = 0.640599, Std. Error = 0.290502, t-value = 2.205, p-value =0.0317, linear regression analysis).

The proportion of gravid female anadromous stickleback was relatively equal to the proportion of nuptially coloured males of the same ecotype at Clachan (Table 3., χ^2= 0.6476, df = 1, p-value = 0.421, Pearson's Chi-squared test with

Yates' continuity correction). The ratio of females to males in breeding condition was even greater for residents although not significant (χ^2= 0.31116, df = NA, p-value = 0.7481, Pearson's Chi-squared test with Yates' continuity correction). A higher percent of resident stickleback were more likely to be in breeding condition than anadromous stickleback, however there was no significance between ecotypes (Table 3, χ^2= 2.1672, df = 1, p-value = 0.141, Pearson's Chi-squared test with Yates' continuity correction).

Numbers of anadromous stickleback caught at Clachan varied with the depth of water from two days prior, with numbers of anadromous caught reducing as water depth increases (Fig. 11). Rainfall between the 1st and 12th May was high (68.4mm in contrast to 32.2mm from the 13th to 31st May, SEPA data for Benbecula), resulting in water depths that were unrelated to the tidal cycle, and these dates were removed from the analysis (Fig. 11). Disregarding the three dates at the start of the trapping period, there is a significant relationship showing variation in females caught with the depth two days previous (Std. error = 0.001, t-value = -4.633, p-value=0.0006, R^2 = 0.641, Generalised linear model). There was no significance between number of females and any other depth or lunar phase. There was also no significant trend in the number of males caught in relation to water depth.
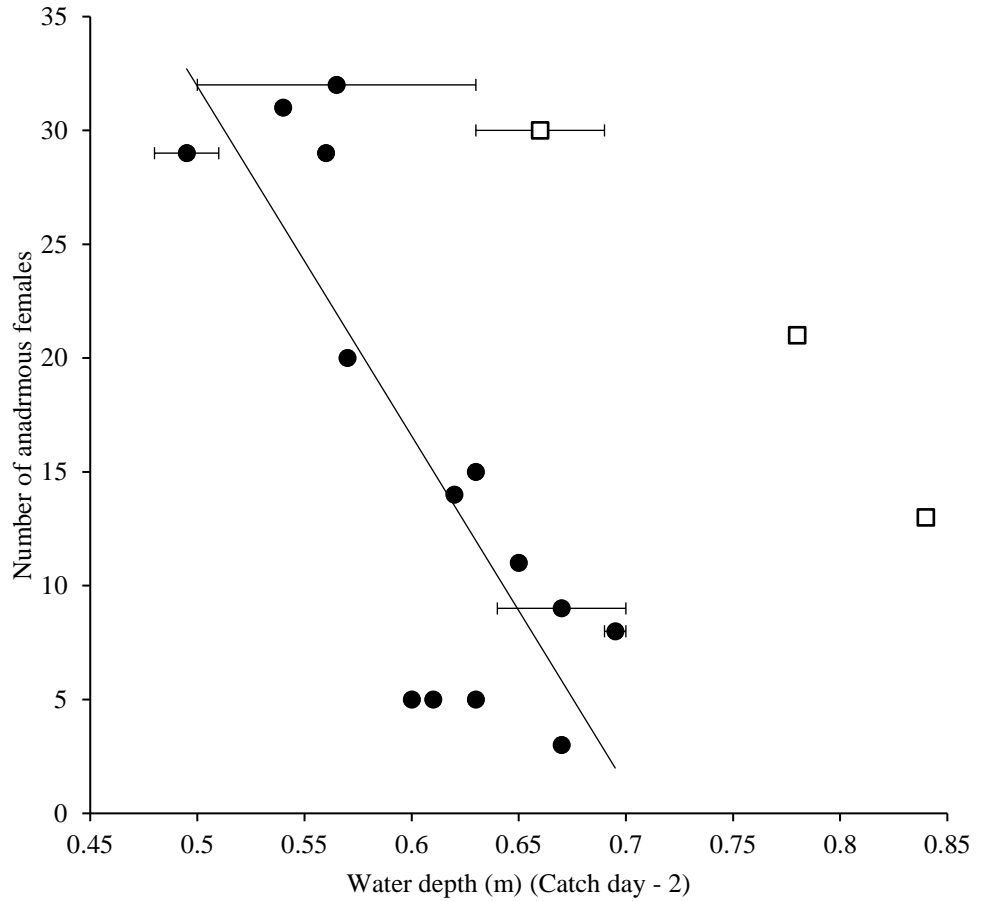
Figure 11. Numbers of anadromous females caught at Clachan-na-Luib and the water depth 2 days previously (e.g. Numbers of fish from 12th May and depth from 10th May). Open squares show values from the first 3 dates of trapping when water depths were high because of heavy rainfall. Filled circles show data from other dates, $R^2 = 0.6452$, 12 df, p-value= 0.000540, linear model. with the square values disregarded. Collection data from the 10th May were not included because of the lack of water depth data from 8th May, dates with error bars did not have a corresponding water depth (-2 days) so an average depth from the two dates closest to 2 days prior was used (Error bars = standard error).

Table 4. Summary for stickleback at Duin. The total number of sticklebacks observed and number of those sampled for standard length and breeding condition (coloured-up or visually gravid).

Ecotype and sex	Total number	Sampled number	Standard length (mm)	Range (mm)	Number of breeding stickleback	Total breeding stickleback in sampled population (%)
Anadromous female	60	25	70.24	60.65 - 81	8	32
Anadromous male	54	22	63.19	57.1 – 72.05	7	31.8
Anadromous unknown	4	0	n/a	n/a	n/a	n/a
Resident female	326	25	36.98	31 – 50 .75	18	72
Resident male	59	20	33.18	29 – 41.9	13	65
Anadromous unknown	9	0	n/a	n/a	n/a	n/a

2.3.3 Clachan-na-Luib and Duin

In all, 512 sticklebacks were captured over the 5 weeks of trapping at Duin. The collection dates at Duin all fall within one day of the main lunar phases (new, half and full moons). Anadromous stickleback equated to 23% of all sticklebacks caught with 77% being residents (Table. 4). This contrasts with what was observed at Clachan (Table. 3). Although the predominant ecotype at both sites was different, the trends seen within the ecotypes were similar. More females than males were caught at both sites (Table. 3, Table. 4 Pearson's Chi-squared test with Yates' continuity correction, χ^2=13.985, p = 0.00018), although the sex difference in the numbers of anadromous stickleback was less at Duin than at Clachan (52.6% and 71.7 % female respectively).

Significantly more female residents were observed than males at Duin (Table 4., χ^2= 185.17, df = 1, p-value < 2.2e-16, Pearson's Chi-squared test with Yates' continuity correction). Male and female anadromous were observed at approximately the same rate (Table 4., χ^2= 0.31579, df = 1, p-value = 0.5741, Pearson's Chi-squared test with Yates' continuity correction). The peak number of observed anadromous females caught at Duin occurred on the 9th June, one day before the new moon on the 10th June (Fig. 12) and the lowest number were caught on the full moon (26th May). This is a similar pattern to that seen at Clachan. However, unlike at Clachan there was no first peak around the 26th May when there was a full moon. The lowest numbers of anadromous females at Clachan and Duin were observed within three days of each other (29th May and 26th May respectively). There was no obvious trend in the number of anadromous males caught at Duin. Male and female resident stickleback at Duin had similar patterns in numbers caught, with both seeing a peak on the 26th May then declining each week, although the numbers of females caught was larger and there was a sharp decline in number of males over the last two weeks not seen in females.
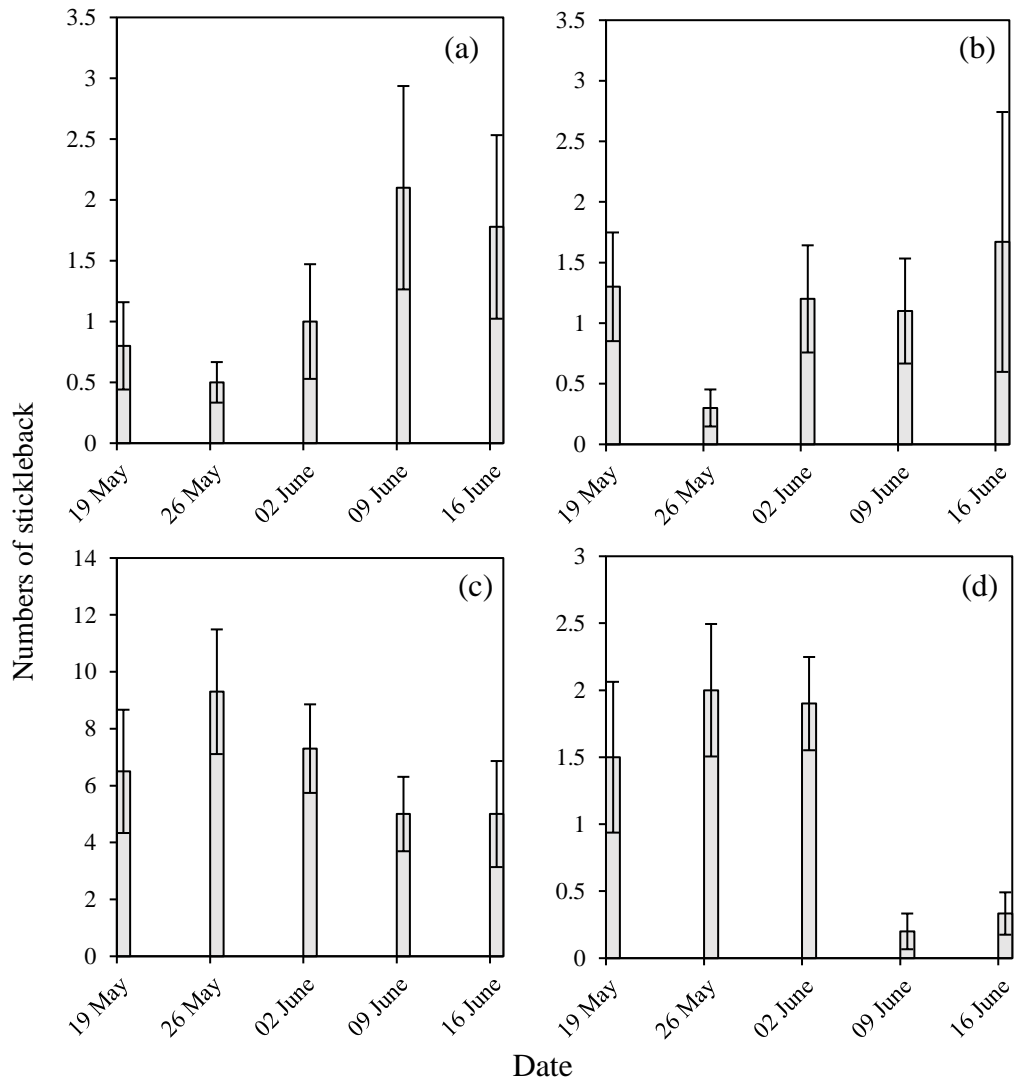
Figure 12. Average number of stickleback of both ecotypes and sex caught per trap between 19th May and 16th June 2021 at Duin. (a)= anadromous females ,(b)= anadromous males, (c)= resident females, (d)= resident males. Error bars = standard error.

There were no obvious overall trends in the size of anadromous stickleback caught at Duin over time (Fig. 14., df=44, p-value= 0.564, Linear regression analysis). Females were significantly larger than males at Duin (Fig. 13., df=44, p-value= 1.15e-06, Linear regression analysis). Anadromous stickleback at Duin were significantly larger than those at Clachan (df=181, p=0.0306, Linear regression analysis). The length of anadromous stickleback also varied by sex and location (df=181, p=0.0202, Linear regression analysis).
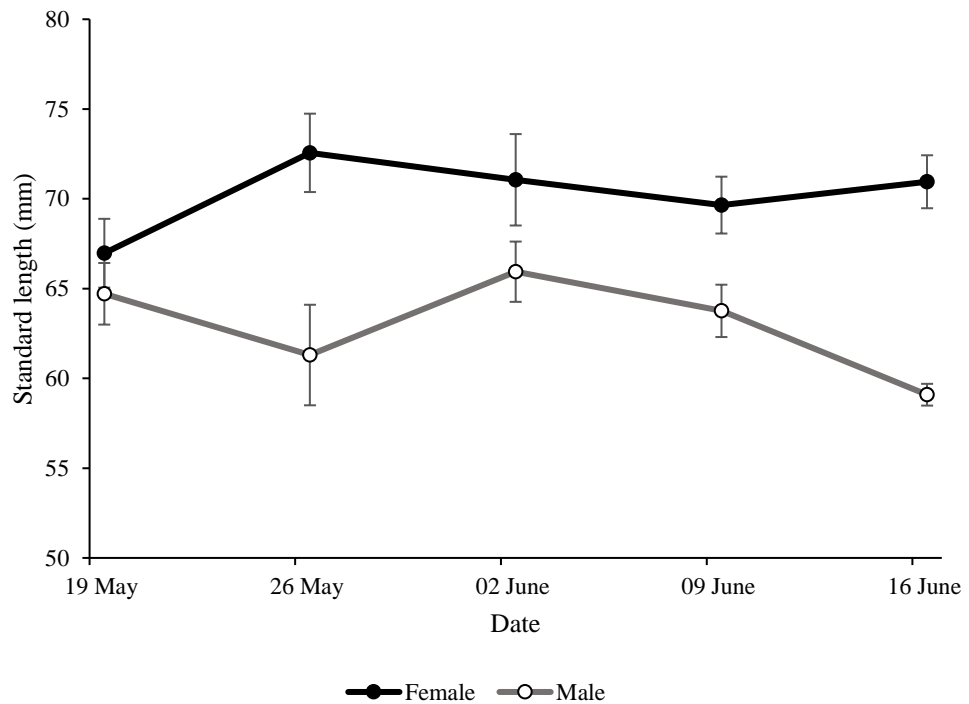
Figure 13. Mean (+SE) standard length of anadromous stickleback at Duin.

At Duin, almost a third (31.8-32%) of anadromous stickleback and around two thirds (65-72%) of residents were in breeding condition (Table 4.). The percent of stickleback in breeding condition was equal between Duin and Clachan when comparing the anadromous stickleback (Table 3., Table 4., $\chi^2 = 0$, df = 1, p-value = 1, Pearson's Chi-squared test with Yates' continuity correction). Comparing residents in breeding condition from Clachan and Duin showed no significant variation (Table 3., Table 4., $\chi^2 = 0.36984$, df = 1, p-value = 0.5431, Pearson's Chi-squared test with Yates' continuity correction).

2.3.4 Predation, disease and parasites.

Blackspot was significantly less common at Clachan than at Duin (Fig. 14, $\chi^2 = 7.3708$, p-value = 0.01949, Pearson's Chi-squared test with simulated p-values) and was absent from resident fish at Clachan ($\chi^2 = 8.0257$, p-value = 0.004498, Pearson's Chi-squared test with simulated p-values). There was no difference in the number of anadromous and residents with blackspot at Duin ($\chi^2 = $

0.3507, df = 1, p-value = 0.5537, Pearson's Chi-squared test with Yates' continuity correction).
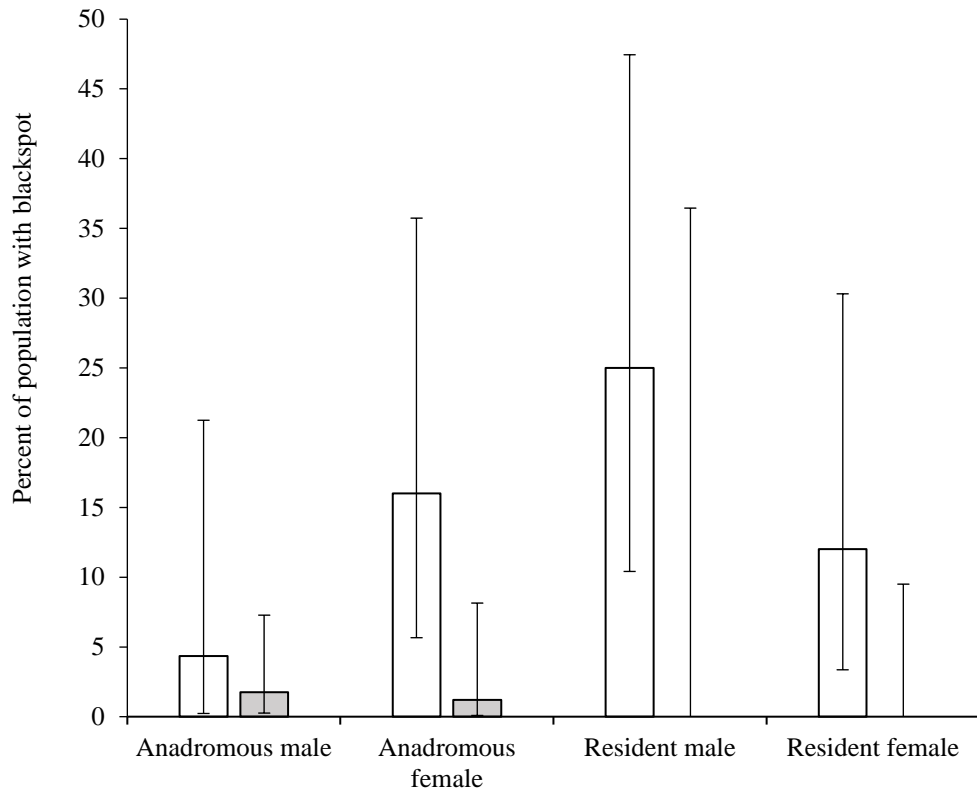
Figure 14. Proportion of male and female stickleback, or the two ecotypes of sampled stickleback with visual signs of blackspot. Open bars are Duin, shaded bars are Clachan. Error bars = 95% confidence limits.

Recording of predation marks began on 1st of June and were recorded on all observed fish from that date, including those of unknown sex. Predation marks on stickleback were only observed on anadromous stickleback and were absent from all resident fish at both sites (χ^2= 8.9708, df = 1, p-value = 0.002743, Pearson's Chi-squared test with Yates' continuity correction). Among anadromous stickleback, those at Clachan that had higher rates of marks of predation (Fig. 15.). Predation marks were more common on anadromous stickleback at Clachan than at Duin, but this was not statistically significant (χ^2= 1.0079, df = NA, p-value = 0.5087, Pearson's Chi-squared test with simulated p-values).
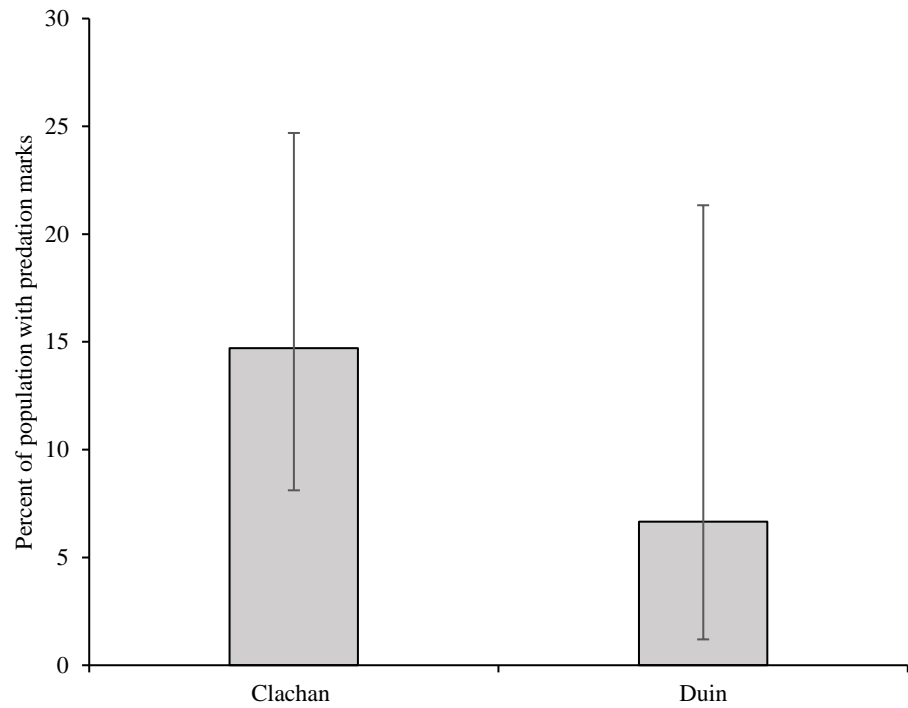
Figure 15. Predation marks ('aviscars') observed on anadromous stickleback at the two sites in June 2021. Error bars = 95% confidence limits.

2.4 Discussion

Few previous studies have investigated the detail of migration in three-spined stickleback. We have shown that much can be learned about this topic using a straightforward approach of frequent censusing of stickleback at two saltwater sites on opposite sides of the Scottish island of North Uist. At both locations, stickleback migrate in from the open sea in spring to breed in saline lagoons, in sympatry with phenotypically quite different 'resident' stickleback that are present in the lagoons year round.

The main findings of this study are that anadromous migration appears protandrous, with males arriving earlier in the breeding season than females. More males arrived in the first half of the trapping period at Clachan than the second. Additionally, males caught at the start and end of the trapping period are shorter in length than those arriving in the middle. Female sticklebacks migrate in two distinct pulses coinciding with the lunar phases, with the length of observed females staying rather consistent. Generally, more females were observed over the whole period than males at both sites. Females were longer than males at both sites. Salinity variation of the lochs did not affect the distribution of anadromous stickleback but did affect the distribution of residents. Marks of avian predation were absent on resident sticklebacks and no discrepancy was observed in number of anadromous sticklebacks with scarring between sites. Blackspot was less common at Clachan, with it being completely absent on resident Clachan stickleback.

2.4.1 Numbers of migrants

Comparing the numbers of stickleback caught between sexes and ecotypes allowed for a better understanding of anadromous migration. There is a large discrepancy in the ratio of males to females found at Clachan, with many more females caught than males. In a study of breeding freshwater stickleback conducted from May to July 1988 in the Tsuya river in Central Japan (Mori, 1993) stickleback were collected and marked and the ratio of male to female sticklebacks was found to be 1:0.7. This ratio is contradictory to what was

recorded at Clachan, which is likely to do with the methods of observation. Mori (1993) isolated a small pool off the main river and likely monitored the whole population within the pool. Our study on the other hand used minnow traps in a wide channel. Having traps set up in a channel rather than isolating an area gives a better understanding of fish travelling through the channel and would be improved by marking individuals caught. This presents two possible explanations of the ratio of male to females seen at Clachan: (1) behavioural factors mean males avoid traps, or females are more likely to enter them; (2) males are less common in this area of the loch.

Boldness is a behavioural trait that encourages exploration and risk taking in an individual, and an increased boldness in stickleback often results in a greater chance of being caught in traps (Biro & Dingemanse, 2008; Álvarez-Quintero, *et al.*, 2021). Previous studies have shown that male stickleback exhibit greater boldness than females, when looking at time spent out of cover exploring their environment (King, *et al.*, 2013). This is inconsistent with the results from Clachan, if boldness correlates with the probability of being trapped. Alternatively, the fact that males are territorial during the breeding season presumably means that they move around only within the constrained area of their territory and avoid 'trespassing' (Van Den Assem, 1967). Therefore, the reduction in number of males caught during the breeding season could reflect a reduction in exploration that comes from defending and maintaining a territory. On the other hand, females are freer to move around the habitat to find a mate. This could lead to higher catches of females.

The reduction in male numbers in the last 3 weeks of trapping may suggest that by this point they were mostly defending fertilised eggs, especially in the last week when numbers dropped considerably. Unlike at Clachan the number of anadromous male and female stickleback caught at Duin was similar. This suggests that it is possible the reason fewer anadromous males were caught at Clachan is the nature of the channel sampled. The sampling site at Duin was quite dissimilar to Clachan: Duin was deeper, wider, had lower salinity and the water was usually still (except at spring tides), whereas at Clachan it was constantly flowing. The sex ratio at Duin suggests that no disparity occurs

between boldness in males and females, and that in a breeding area that is not a channel the number of sticklebacks caught might be dictated by the total population. Although there was little discrepancy between sexes of anadromous stickleback at Duin, numbers of resident females significantly outweighed males at a ratio similar to that observed at Clachan. It is possible that the numbers of resident males recorded were lower than the numbers of anadromous males, because anadromous stickleback are often more aggressive than resident stickleback (McKinnon, *et al.*, 2012), and therefore might increase probability of being caught.

The distribution of stickleback ecotypes at Clachan and Duin was associated with salinity, with more residents being more common where salinity was lower. Anadromous stickleback on the other hand, did not have preference for a particular salinity. Salinity is known to play a role in the dispersal of stickleback, with anadromous and freshwater stickleback having a preference in salinity closest to that which they are used to (Fryxell & Schlutz, 2012). This is consistent to what was observed with the distribution of resident stickleback at Duin and Clachan, although is not apparent in anadromous stickleback.

2.4.2 Temporal trends in migration

The timings of migration of anadromous stickleback varied between sexes. In the six week period of observations, the recorded numbers of female anadromous stickleback increased and decreased in two distinct pulses, whereas males steadily declined in numbers over the trapping period. The first 3 weeks a higher number of males and females were recorded than in the second 3 weeks, although only males had significantly more. Stickleback are known to migrate into lakes and lagoons from April in the Baltic Sea (Bergström, *et al.*, 2015). Migrating earlier in the breeding season increases the chances of breeding success (Morrison, *et al.*, 2019). Increased breeding success is likely the reason for a higher catch rate in the first half of the study, especially in males who require time before mating to establish a nest.

Pacific Ocean anadromous stickleback observed at two sites in Japan, Akkeshi Bay and Obetsu River, 5 Km apart (Kume *et al.*, 2005) were observed to have between one and two peaks of migration. The study showed a time lag in the peaks, with the site location closest to the Pacific Ocean occurring first. Although similar to the study on North Uist, the peaks in Japan do not correlate to the new and full moons from that point in time. The same study in Japan showed that in some years the Pacific Ocean stickleback have only a single peak in numbers while the closely related Japan Sea stickleback (*G. nipponicus*) there are three separate peaks.

2.4.3 Tidal cycles

Within the pulses of female migration there was a relationship between numbers caught, water depth, lunar phase or both. Lunar phase drives the tidal cycle, and thus it is difficult to be certain whether fish are responding to (changes in) water depth, strength of tidal currents or the light from the moon. In addition, spring tides are known to bring about the lowest levels of turbidity as clearer seawater predominates over turbid freshwater (Giri, *et al.*, 2019). However, the patterns in numbers migrating suggest that some cues are more important than others. In particular, the number of anadromous females caught declined as the depth of water in the recent past (two days previously) increased. Migration is known to be energetically straining upon many anadromous fish and smaller migratory fish experience greater selection to conserve energy for reproduction (Crossin, *et al.*, 2004; Jonsson & Jonsson, 2006). Bernatchez & Dodson (1987) showed that among anadromous species, increasing length of migration was related to increasing body length and energy efficiency. Fish are thought to choose the migratory pathway that minimises energy expenditure by zigzagging across river channels to take advantage of weaker currents (McElroy, *et al.*, 2012) and avoiding areas of high flow (Jonsson, *et al.*, 2018). Additionally, juvenile fish are known to "ride the tides" to reduce energy expenditure (Krumme & Saint-Paul, 2010). It can be assumed that female stickleback would save as much energy as possible for

breeding, therefore it is likely that migration would occur when there is least resistance in water velocity. In our data it appears that in general more female anadromous stickleback migrate as the tides move towards spring, and may be taking advantage of the currents to move inland after the low tides.

The start of May saw the highest level of rainfall over the whole trapping period, associated with the higher number of anadromous females that were caught at the start of the trapping period. These dates were mostly after the first new moon, when the numbers of anadromous females might have been expected to be low. It is possible that the reason for the increase in numbers during this period is a result of the high rainfall and correspondingly deeper water in the channel at Clachan. This seems surprising, as an increase in water level at other times was associated with a decrease in the numbers of anadromous females. Heavy rainfall is known to alter stream flow patterns and influence timings of migration in salmonids (Kelson & Carlson, 2019). The rainfall at the start of May likely altered the rate of flow at the channel at Clachan, which increased the numbers of anadromous females being caught. High rainfall often acts as a driver in migration for salmonids and neotropical fishes (de Magalhães Lopes, *et al.*, 2018; Kelson & Carlson, 2019), this driver could be emulated in anadromous female stickleback.

2.4.4 Illumination

The days after the new and full moon have a sharp decline in numbers of females caught at Clachan, suggesting a direct relationship between these moon phases and numbers migrating. Previous studies have evidenced an association between the illumination of the moons and anadromous migration. The new and full moon often cause the darkest and lightest nights respectively. Illumination in anadromous migration appears to change with variations in the size of the migrant, with larger species preferring higher illumination. The relatively large lake sturgeon (*Acipenser fulvescens*) showed a strong association between migration and the time leading up to a new moon and during and just after the full moon (Forsythe, *et al.*, 2012), whereas smaller

species, anadromous herring and eels showed higher levels of migration when visibility in the water was low around the new moon (Yako*, et al.*, 2002; Sudo, *et al.*, 2014). Risk of predation increases with the illumination of the full moon (Longland & Price, 1991), so smaller fish more vulnerable to predation would likely migrate at periods when predation risk is reduced. Female anadromous stickleback at Clachan although affected by the lunar phase, do not seem strongly affected by lunar illimitation. Females were caught in high numbers up to the day before the new and full moons, suggesting that increasing or decreasing levels of illumination has no significant effect on migrating females.

Trends of anadromous females at Duin also show pulses associated with lunar phase. The results from Duin further demonstrates the effects the new and full moon has on numbers of females. At both sites, any collection dates that fell on or the day following the full and new moons had lower catch rates that day previous. Potentially, trapping data from before the full moon and on the new moon from Duin would observe results similar to that which was seen at Clachan.

2.4.5 Protandry

The higher numbers of males seen at the start of the observation period supports the idea of protandry in anadromous stickleback. This is further reinforced when looking at the decline in the numbers caught over the observation period. Protandrous migration in fish is not well researched, although it has been noted to happen in Pacific salmon (*Oncorhynchus spp.*) (Morbey, 2000). There are two long standing hypothesis postulating the factors that cause protandrous migration. The "Mate-opportunity" hypothesis suggests that an earlier arrival time enhances reproductive success through maximising the mating opportunities (Wiklund & Fagerström, 1977). The "Rank advantage" hypothesis, claims that the selective process driving sex-based migration is gaining the best territories (Ketterson & Nolan, 1976).

At Clachan smaller males arrived earlier in the breeding season than larger males. Female size remaining constant over this period suggests that size dependent migration is linked only to male stickleback, although other studies have shown the size of reproductive anadromous females declining during the migratory period (Karve, *et al.,* 2013). It can be assumed that the early migration of smaller males is related to the increased reproductive success, and larger males may delay migration until gravid females start to appear and the predation risk from terns has decreased (Candolin & Voigt, 2003). Many studies have shown female stickleback to choose a mate based on a male's body size, shape, nuptial colouration and water scent (Reimchen, 1989; Bakker & Mundwiler, 1994; Rafferty & Boughman, 2006; Head, *et al.*, 2013). However, nest location has been shown to be a stronger determining factor in mate selection than morphological factors (Sargent, 1982; Bolnick, *et al.*, 2015, Dean et al 2021). With location, depth and quality of the nest significantly influence breeding success. Previous studies have shown male stickleback have a heritable preference for variation in nest location and depth (Vines & Schluter, 2006; Southcott, *et al*., 2013). With nests being such an essential factor in breeding success, the importance of early migration for obtaining premium nest locations for smaller males cannot be understated. This supports the "rank advantage" hypothesis (Ketterson & Nolan, 1976) as smaller males increase reproductive success by selecting better territories earlier and is evidence for protandry based migration in anadromous stickleback. There was no obvious decline in the numbers of anadromous males at Duin with an approximately constant number of males seen each collection.

2.4.6 Evidence of site fidelity

Standard lengths varied between ecotypes, sexes, locations and dates. Anadromous fish were substantially longer than resident fish. This suggests a major benefit of migration: that nutritional conditions are much better in the sea than in freshwater. This is likely to arise partly as a result of higher abundance of (especially planktonic) food in the sea, coupled with less

seasonal variation, as well as greater nutritional quality (Habib, *et al.*, 1997; MacAvoy, *et al.*, 2009). A major difference between marine and freshwater is the substantially greater abundance of fatty acids in the sea, which are critical components of the diets of fishes, both as organic constituents and sources of metabolic energy (Twining, et al., 2021). In common with most stickleback populations, anadromous females tended to be of greater size than males, but by a greater margin at Duin than at Clachan. This may reflect better feeding conditions in the rich waters of Loch nam Madadh, than those that are available to Clachan fish on the west side of Uist (MacColl, *et al.*, 2013). The fact that residents at Duin were smaller than those at Clachan, suggests that the anadromous size difference originates outside the lochs.

The catch rate at Duin suggests a possible migratory pulse twice as long in duration as that which was observed at Clachan. At a much larger spatial scale, variations in the downstream migration observed in salmon of two populations in Alaska and British Columbia show a contrast in the factors that influence migration with the main varying trait being one population being affected by lunar period and flow rate and not the other (Spence & Dick, 2014). It is highly unlikely however that on such a small spatial scale over North Uist that there would be this sort of variability in migratory factors in female stickleback.

Site fidelity within anadromous stickleback is little researched. There is evidence of site fidelity in anadromous stickleback, although confirmed only up to a 300m displacement (Inanova, *et al.*, 2019). More research is needed to determine if site fidelity occurs at greater distances.

2.4.7 Predation

There was a clear difference in the number of avian beak marks ('aviscars' Reimchen, 1988) upon resident and anadromous stickleback. Predation occurred at the same rate between anadromous stickleback regardless of site. Both sites had no occurrences of predation marks on resident stickleback. Stickleback migratory timings and routes may coincide with minimal predation

risk (Brönmark, *et al.*, 2008). The most likely avian predator is terns (*Sterna* spp.), which arrive to breed at approximately the same time as stickleback start to migrate into lochs (Candolin & Voigt, 2003). They are often seen diving for fish over both coastal waters around Uist and the inland lagoons. Up to a maximum 90% of predation attempts upon stickleback result in failures (Reimchen, 1991a), which is often due to the presence of pelvic and dorsal spines (Hoogland, *et al.*, 1956). Thus, there are two possible interpretations of the pattern we recorded: either both ecotypes are attacked, but only anadromous fish manage to escape, or resident fish are seldom attacked. Anadromous fish have much more developed armour, so probably do escape from attack frequently, but it is notable that there is a sharp distinction in fear of overhead attack between the ecotypes. Anadromous fish in a bucket respond to something overhead with panic, whereas resident fish seem oblivious. This suggests that resident fish are seldom attacked by birds and suggests that avian predation risk may be a significant cost of migration.

The size difference between the ecotypes may explain the difference in probability of being targeted. Reimchen's (1988) study of avian predation upon freshwater stickleback showed a correlation between body size and expectation of scars. Stickleback of length <50mm (the size of residents) were absent of aviscars, they were a rare occurrence between 50-70mm (the size of most anadromous) and regular on stickleback >70mm.

Once a stickleback is captured by a predator it is often subject to manipulation to a headfirst orientation before swallowing (Reimchen, 1991b, Reimchen, 1994). These manipulations can often lead to failure and the stickleback escaping, the repeated manipulation by a beak leaves multiple scars. Most anadromous stickleback found to have aviscars in North Uist had multiple scars evidencing manipulation. The large body size and the armour plating of anadromous stickleback substantially helps with escaping from predators and increases the likelihood of survival post-attack (Hoogland, *et al.*, 1956; Reimchen 1992; 1994).

In contrast to possible differences in predation there were no obvious differences in parasitism between the ecotypes, at least for visible ectoparasites. We originally attempted to monitor the monogenetic trematode, *Gyrodactylus arcuatus*, which is common on fish in saltwater around Uist, but this turned out to be unreliable with living fish in the field. Blackspot, which are encysted digenean trematodes of an unknown species (possibly *Podocotyle* sp), were present, and although more common at Duin, did not differ in occurrence between the ecotypes. The difference between sites, but not ecotypes is consistent with the idea that anadromous stickleback return to spawn at the same location where they were born.

2.4.8 Conclusion

Similarities in data were observed between the two lochs, both having females being larger than males at both sites with the males declining increasing then declining in size over the trapping period. Similar migratory trends were observed at both sites, however Clachan showed a more consistent pattern likely due to the more frequent observation days. The frequency of ecotypes observed differed between sites, with more anadromous being observed than residents at Clachan and the opposite at Duin. Blackspot was also less common at Clachan than at Duin.

The timings of migration of anadromous stickleback appears to be sex dependent, with male migration being protandrous and female migration being dependent on environmental cues. Smaller male anadromous stickleback migrate earlier in the breeding season, likely in order to establish a nest in advantageous territories. Larger males migrate later, as reproductive success is increased with an increased body size, so they can migrate later when there are more females and possibly fewer predators. Females migrate indiscriminate of length and dependent on the lunar cycle. The period after the half-moons to before the new and full moons possibly presents ideal migratory conditions for females to conserve energy for reproduction. There is evidence to suggest that other factors such as, rainfall, turbidity and visibility are likely influential in

migratory timings, and further work would be necessary to further disentangle the mechanisms of migration in anadromous stickleback.

3. Chapter 2

Efficiency and sampling bias of an ROV for estimating population density of three-spined stickleback (*Gasterosteus aculeatus*)

Abstract

With the increased pressure on aquatic species from anthropogenic factors the need for constant monitoring of populations is paramount in conservation and management efforts. The development and accessibility of personal remotely operated vehicles (ROVs) in recent years has led to an increase in the use of ROVs in population density surveys. We applied the use of a ROV alongside minnow traps to assess population densities of three-spined stickleback (*Gasterosteus aculeatus*) in five lochs across North Uist, Scotland. Our study had three main findings; (1) Catch per unit effort was higher with the ROV than minnow traps; (2) Anadromous were observed at a higher rate than residents when using the ROV; (3) Vegetation affects the reliability of results when using the ROV. Sampling bias was observed in the ROV as a result of behavioural reactions by stickleback and the vegetation cover of a habitat. Our results suggest that ROVs be used alongside minnow traps to give a more reliable estimate of stickleback population density.

3.1 Introduction

The conservation and management of wildlife are becoming increasingly important in a world of increasing environmental pressures caused by anthropogenic sources. To develop effective conservation and management solutions for a particular species it is important to have an idea of population, and whether it is increasing or decreasing. For estimating population densities of small fish, there are limited methods; trapping, netting and electrofishing (He & Lodge, 1990; Ruetz III, *et al.*, 2011). Each of these methods can produce a bias that influences the numbers of fish caught and can result in poor estimates of population density. Both trapping and netting can lead to a bimodal distribution of catch rates, with individuals moving towards traps and nets with conspecifics already in (Finstad & Berg, 2011; Kressler, *et al*., 2021). Electrofishing is also subject to bias, with the method often causing specific species of fish to flee (Bovee, 1982). These methods may give accurate estimates of catch per unit effort (CPUE), but it is difficult or impossible to convert these to population density estimates.

Developments in technology have constantly changed the way wildlife surveys are conducted. To document populations of elusive (e.g. low density, shy or nocturnal) terrestrial species, remotely triggered cameras ("camera traps") are often used. These are stationary and take a picture or recording whenever a sensor is triggered (Williams, *et al*., 2014). It remains difficult to estimate density from such point recording. The most practical and reliable kinds of population density estimation employ variations on transects (Edgar, *et al.*, 2004), which requires observers to move. Constantly developing technology, means that cameras can now be attached to unmanned remote vehicles for surveying large areas.

Drones have become more commonplace as helpful tools and allow for observations that were previously unobtainable. Aerial drones have long been used in a variety of sectors, including within construction, military, journalism and for transporting medicine (Kardasz, et al., 2016; Chamberlain, 2016; Ackerman & Koziol, 2019; Li & Liu, 2019). Aerial drones for terrestrial

surveying offer a more cost-effective, safer, and less intrusive solution to species and habitat monitoring than the alternative of satellite-based remote sensing and manned surveys (Broich, et al., 2011; Christie, *et al.*, 2016). Aerial drone technology is now well established, along with its use for surveying landscapes and wildlife populations. In contrast, economically accessible underwater drones have only recently become available, and their use in research has been poorly explored.

The development of remotely operated 'underwater' vehicles (ROUVs or more commonly ROVs) has advanced significantly in recent years. This advancement has seen an improvement in drone technology and functionality and reduction in cost, which in turn has seen a year-on-year increase in the use of ROVs for research since the early 2000s (Sward, et al., 2019). Just over a decade ago (2008) ROVs costing between \$10,000 - \$100,000 were classed as 'low cost', relative to previously available technology (Sward, et al., 2019). Now small ROVs can cost as little as \$1,000.

Underwater drones have already been used in research surveying, e.g. for quantifying vegetation cover (Donadi, et al., 2020) and distribution of community species on slopes (Soares, et al., 2022). Additionally, drones have been used in the observation of a wide variety of targeted fauna, including teleost fish (Haggarty, et al., 2016), elasmobranchs (Henry, et al., 2016) and cephalopods (Zeidberg & Robinson, 2007). To our knowledge all previous studies using ROVs have been conducted on organisms larger than stickleback (~3-7 cm).

ROVs can be used in a similar way to underwater visual censuses (UVC) to conduct surveys of species at shallow water depths. When compared to a diver's eye there are limitations of the resolution and field of view of ROVs (Andaloro, et al., 2012). However, being able to observe the camera recording, depth and heading on the same screen, whilst maintaining a constant speed on a single plane, gives the ROV an advantage over human diving. Furthermore, ROVs are capable of operating at depths beyond that of safe scuba limits (~30m) and can be approximately equal in cost to scuba equipment (Sward, et

al., 2019; Raoult *et al.,* 2020). A previous study comparing the use of ROVs and snorkelling for video-based research on Butterflyfish (*Chaetodontidae spp.*), showed the benefits of ROV based surveying (Raoult, *et al.*, 2020). A higher abundance and species richness was recorded using an ROV and fish behaviour did not differ between using the ROV and snorkelling. Comparing ROVs and diving in shallow habitats, differences in abundance were shown depending on rockfish species (Carpenter & Shull, 2011), attributing the bias to fish behaviour. The diver was able to observe in crevices that the ROV was not able to, areas which some species frequent. Comparatively, other studies have shown that divers produce a greater estimate of species abundance than ROVs which more frequently miss cryptic species (Pita, *et al*., 2012). Manned submersibles are also used for population surveying at greater depths. Reaction to ROVs have been observed to be five times greater than the reaction to larger manned submersible in smaller species, with reactions being defined as a distinct movement in change of course or swimming speed (Laidig, *et al.*, 2012). It appears evident that the use of ROVs when compared to diving methods for surveying populations yields a lower estimate of abundance. However, the ROVs is still a useful surveying tool for observing species that are less elusive. Therefore, for observing and estimating populations of anadromous stickleback, ROVs might be suitable.

This study will compare the use of an ROV to minnow traps for the monitoring of three-spined stickleback (*Gasterosteus aculeatus*). Furthermore, it will assess whether underwater drones have the capability to be used as a primary method for underwater surveys of stickleback populations and assess the wider possibilities relating to underwater drone capabilities.

3.2 Methods

We compared the CPUE of stickleback recorded in video footage by the drone while doing transects, with the CPUE from using minnow traps. The ROV used for the underwater surveying during this study was the GLADIUS MINI Underwater Drone with a 50m cable (Fig. 1). The drone must be constantly tethered to the above water controller and connected to a smartphone or device with Wi-Fi compatibility.

Figure 1. GLADIUS MINI Underwater Drone used for surveying stickleback across North Uist (Chasing, 2022).

Five sites were sampled in June 2021 across North Uist, Scottish Western Isles, two were lochs that connected to the sea and three were isolated freshwater lochs (Table 1., Fig. 2). The two lochs connected to the sea were an-Duin and Hosta (hereafter Duin and Hosta) the latter of which is freshwater, and the three isolated freshwater lochs were Scadavay, Tormasad and a'Bharpa (Hereafter Scad, Torm and Bhar). The sites were chosen because they are known to have large variation in CPUE based on trapping.
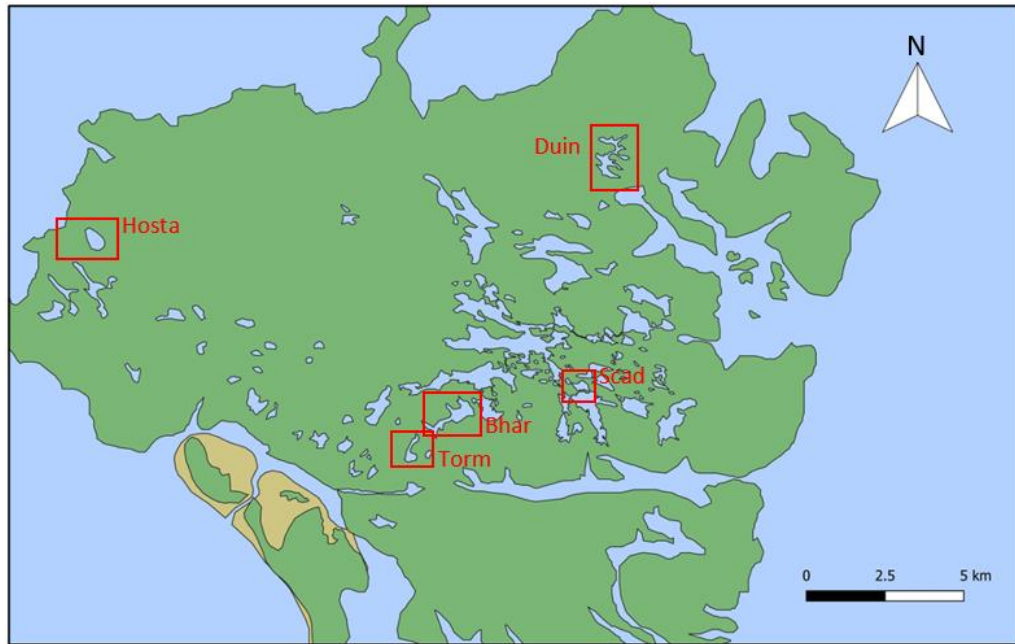
Figure 2. Map of North Uist with the locations of the five lochs sampled for stickleback with traps and the ROV.

Table 1. Location of surveyed sites on North Uist, giving the latitude and longitude (Garmin GPSMAP 64s) of centre of the area where transects were conducted and minnow traps set.

Loch	Coordinates
Duin	57°38'34.9"N, 7°12'54.1"W
Hosta	57°37'40.3"N, 7°29'13.3"W
Bhar	57°34'14.4"N, 7°18'06.4"W
Scad	57°35'04.8"N, 7°14'09.9"W
Torm	57°33'43.4"N, 7°19'05.2"W

Initially the intention was to use the drone for straight line strip transects zig-zagging perpendicular to the shoreline for 5 minutes and repeated three times. After initial test runs it appeared that the drone was incapable of travelling in a

completely straight line because of water currents and unreliability of the internal compass when submerged. To resolve this and keep the test consistent, we opted to carry out surveys by steering the drone directly ahead (with the exception of avoiding obstacles) and not attempting to correct changes to the heading caused by currents. This method may have resulted in limited re-crossing of survey areas. The battery capacity of the drone also limited the transects to two per location.

Two people were required to undertake surveys using the drone, one as the pilot and the other to feed the cable out of the spool to prevent tangling of the line. The need for a second person might be eliminated by the use of a Reel or an E-Reel (Automatic Reel). The 50-metre cable was sufficient for a transect of 5 minutes, however longer durations of transect would likely require a longer cable. The drone was unable to record speed, although the speed of the drone was kept low and constant, in order to get good quality video footage. The drone was kept at a depth around 0.2-0.3m off the substrate. At depths <0.3m from the surface the ROV recorded substantial amounts of movement due to the water surface motion, with the movement increasing closer to the surface. All transects used the ROVs lights which aided with visibility, although they were not needed at Duin and Hosta. Learning how to pilot the ROV with the combination of the controller and a mobile device was intuitive, however to pilot perfectly requires a steep learning curve. Maintaining a constant speed and enough height over substrate and vegetation was difficult because of the sensitivity of the controls.

The catch from un-baited minnow traps was used as a comparison for the number of sticklebacks seen in the drone footage. After the transects were carried out, five traps were set for a period of 24 hours, evenly spaced over the same area that was surveyed with the drone. When traps were lifted, the contents were emptied into buckets containing aerated loch water, counted and returned to the loch. Stickleback caught in the traps at Duin were noted for ecotype and then released. Two ecotypes were recognised, anadromous and resident, determined by an individual's size and armour plates.

Drone footage was downloaded onto a computer and observed. Whenever a stickleback was recorded it was noted alongside the timestamp and ecotype if it could be determined. Time spent by the drone passing over different types of substrate (vegetated versus unvegetated) was also recorded. An impression of the clarity of the water at each loch was also noted. Comparing CPUE with vegetation cover was not undertaken for Scad, this was because of no observations of stickleback being made and no variety in vegetation being observed.

CPUE in the minnow traps was calculated as mean stickleback caught per trap per day for each site. ROV CPUE per hour for the ROV using the mean of the two transects. The relationship between CPUE (ROV) and CPUE (traps) was quantified with a Linear regression. Chi-squared tests were used to test whether the proportion of anadromous and resident fish in Duin differed between ROV and trapping data. A Chi-squared test was also used to test whether sightings of fish from the ROV were independent of the substrate type (vegetation).

3.3 Results

During all ROV transects no other fish species other than three-spined stickleback were observed. While using the ROV, foraging was observed in two anadromous sticklebacks at Duin. Additionally, a reaction of bolting and hiding was only observed in residents. Because of the low numbers of stickleback observed and the nature of stickleback to shoal together, quantifying stickleback reactions was impossible.
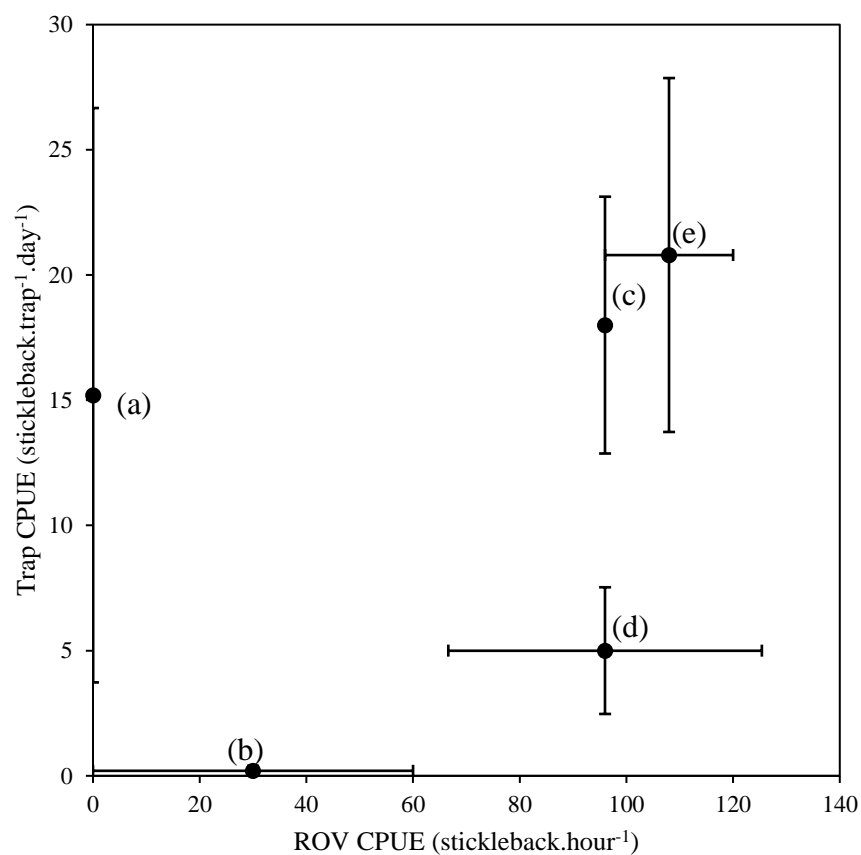
Figure 3. CPUE of stickleback using the ROV and minnow traps, (a) Scad, (b) Torm, (c) Duin, (d) Bhar, and (e) Hosta. Error bars = standard error.

There is no real correlation between CPUE with each method at each site (R^2= 0.1065, p-value= 0.592, Linear regression model), with the results from Scad removed this correlation does improve but only moderately (R^2= 0.6143, p-value= 0.2162, Linear regression model). The estimates of CPUE appear to be more consistent for higher density sites (Hosta). The CPUE was highest in

Hosta for both the ROV and minnow traps (Fig. 3). It is especially noticeable that the CPUE recorded from trapping was moderately high in Scad, but no stickleback were sighted from the drone. In the majority of traps at Scad a low number of sticklebacks were caught (n=2,3,4,6) but in one trap a much larger number were caught (n=61). Duin, Torm and Bhar show a somewhat reversed pattern to Scad (more fish recorded with the drone).
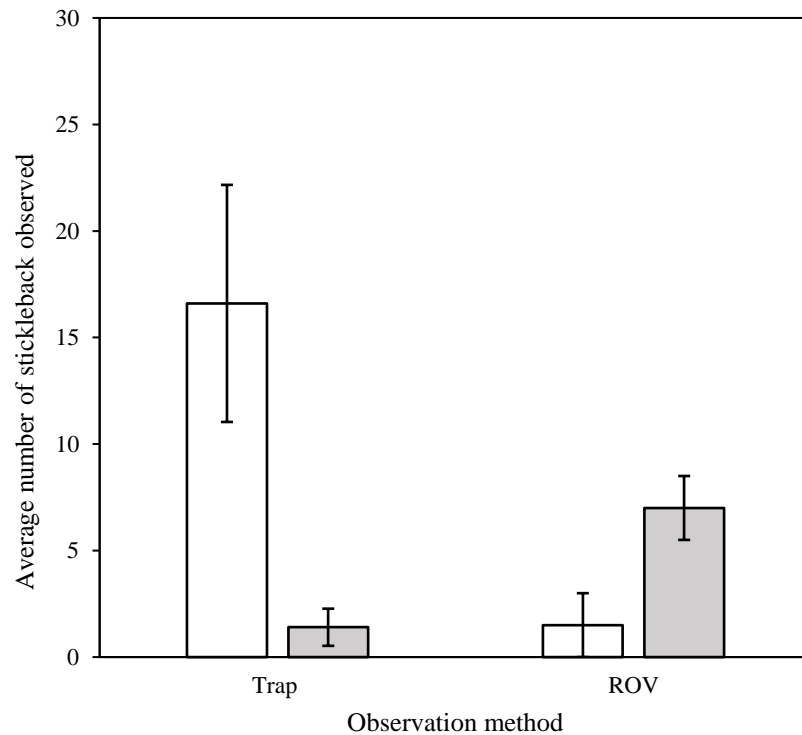
Figure 4. Comparison of average number of different ecotypes caught/ observed per trap and ROV transect at Duin for all 5 traps and 3 transects. Unfilled bars represent resident stickleback, grey bars represent anadromous (Error bars = Standard error).

The numbers of stickleback caught in the minnow traps at Duin were dominated by residents (n=83), with few anadromous being caught in comparison (n=7). This was different to what was observed through the drone, with significantly more anadromous stickleback being seen than residents (Fig 4). The proportions of anadromous and resident stickleback caught using the trap and observed from the ROV were significantly different (χ^2 = 51.89, df=1, p-value = 0.0005, Pearson's Chi-squared test with Yates' continuity correction).

| | Duin High vegetation 419 seconds | Duin Low vegetation 181 seconds | Hosta High vegetation 90 seconds | Hosta Low vegetation 510 seconds |

| | Bhar High vegetation 88 seconds | Bhar Low vegetation 512 seconds | Torm High vegetation 354 seconds | Torm Low vegetation 246 seconds |
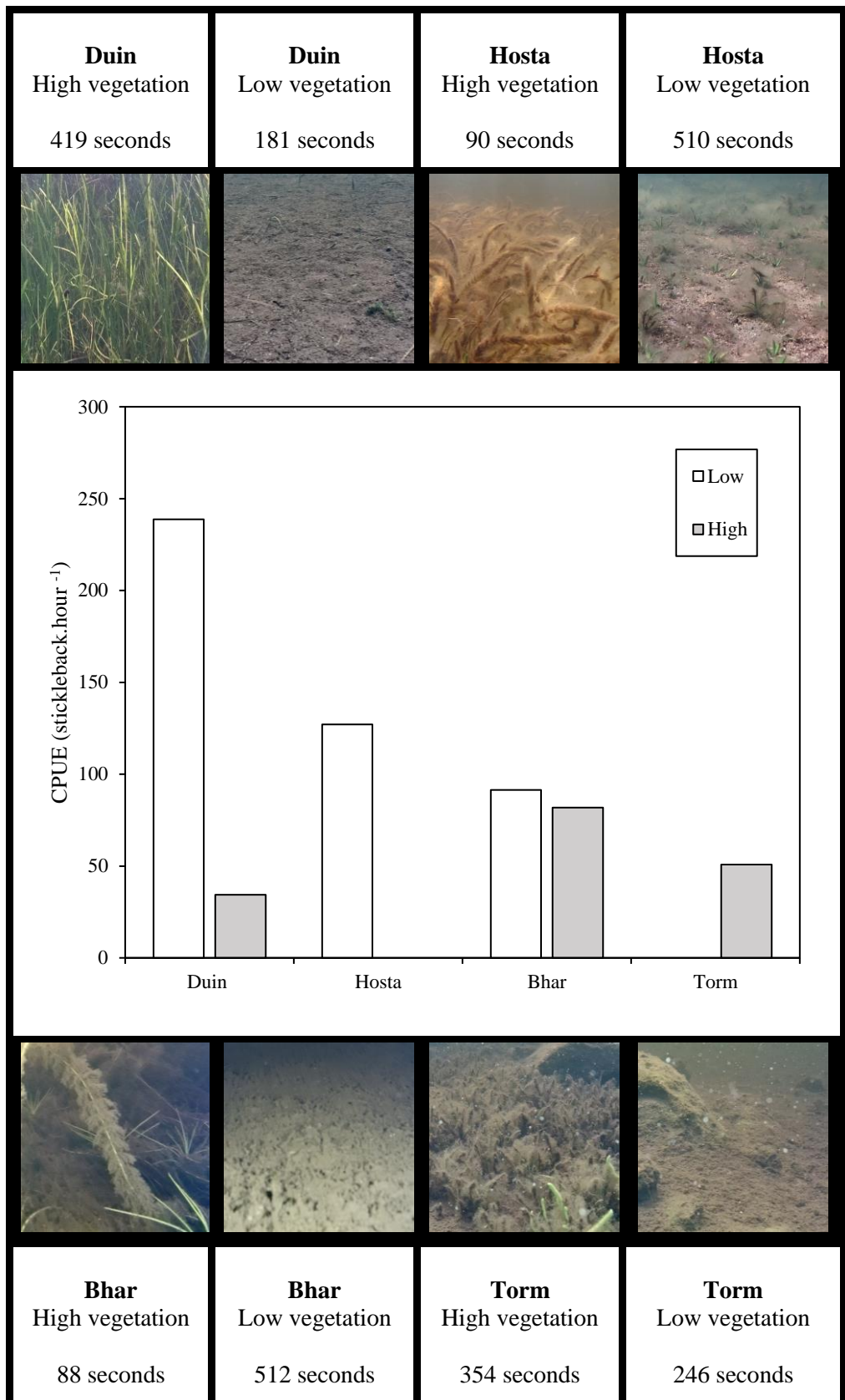
Figure 5. The CPUE of stickleback in areas of low and high vegetation, observed using the ROV. Examples of high and low vegetation and the time spent over each are shown in panels at top and bottom.

Duin and Hosta had high visibility and high vegetation cover, with a low level of suspended particles (Fig. 5). Torm had an intermediate level of visibility, vegetation cover and suspended particles. Visibility and vegetation were low at both Scad and Bhar, and both had high numbers of suspended particles. The type of vegetation varied between the five sites, with substantial differences in species, height and density. Vegetation was highest at Duin, where there were areas of tall, dense, sea grass (*Zostera*) beds, but other areas were bare mud, completely devoid of visible plant life. Hosta had almost complete vegetation cover throughout the transects, only varying in plant height and density. The majority the loch floor at Hosta was low vegetation consisting of short plants covered in algae. Torm had the lowest plant height of vegetation in the 'high vegetation' category with the height of plants being approximately <20cm. The plants at Bhar and Scad were very sparse, likely a result of the low light penetration from suspended particles. The high vegetation at Torm and Bhar allowed for the substrate to be always observed due to the general height and sparseness of the plants.

At Duin, stickleback were disproportionately associated with areas of high vegetation (Fig 5), but there was no pattern in other lochs. There was significantly fewer stickleback observed in the areas of low vegetation at Duin ($\chi^2 = 12.551$, d.f.=1, p-value=0.0003961, Pearson's Chi-squared test with Yates' continuity correction). None of the other sites had a significant difference in the numbers of stickleback observed in different vegetation (Hosta: $\chi^2 = 3.1602$, p-value= 0.09195; Bhar: $\chi^2 = 0.058642$, p-value=1; Torm: $\chi^2 = 3.4547$, p-value=0.08046, Pearson's Chi-squared test with simulated p-values). Comparing CPUE in low vegetation, Hosta had just over half the CPUE of Duin (Fig. 5), although had the highest CPUE overall (Fig.3).

3.4 Discussion

The CPUE per trap per day was less than the ROV CPUE per hour, when looking at the five sites combined. When looking at the individual sites, there was no real correlation between CPUE of each observation method, although there was a moderate corelation without Scad. At Duin, which contained both anadromous and resident stickleback, there was a significant difference in the dominat ecotype with the two methods. The ROV had a higher CPUE for anadromous, whereas minnow traps CPUE was high for residents. The vegetation height and density were shown to be an influencing factor of stickleback distribution at Duin, with far more stickleback being observed in areas of low vegetation.

The observations made using the ROV look to be influenced strongly by the ecotype of stickleback as well as the vegetation height and density. The behaviour of stickleback differs between ecotypes and therefore might affect the rate of observations using the ROV.

3.4.1 Limitations of ROV observations

The presence of dense and tall vegetation made observing stickleback more difficult, firstly because vegetation affected vision and secondly because it offered refuge for stickleback trying to avoid the ROV. Only when stickleback are swimming near the top or above of the vegetation are they easily visible. Vegetation also hindered the ability to navigate the transects. Tall vegetation presents a physical obstacle that had to be avoided, moving the ROV to a height that made observations harder because of visual distance limitations of the ROV. Additionally, vegetation had the ability to become caught in the propellors of the ROV causing a momentary delay in transects.

At Duin, using the ROV whilst moving over dense, tall vegetation resulted in fewer sticklebacks being observed and most that were being anadromous, suggesting that resident stickleback possibly retreated into the vegetation after

seeing the ROV or already are in the vegetation. Most observations were made on the muddy substrate and were again mostly anadromous. This was to be expected as during the breeding season resident lagoon stickleback tend to build nests on weeds, whereas anadromous preferred sand or gravel substrates (Dean, *et al.*, 2021). The results from Duin would produce different estimates in population densities of sticklebacks, one having a higher proportion of anadromous (ROV) and the other a higher proportion of residents (Minnow traps). This demonstrates the bias in ROVs but also demonstrates the flaws in the current method using minnow traps.

At Torm, stickleback were only observed in areas of "tall" vegetation, although this "tall" vegetation was the shortest of all lochs and was not too dissimilar to the "low" vegetation observed in Hosta. Suggesting the idea that stickleback, especially residents, are more commonly found in areas of densest and highest vegetation and ROV observations are limited by the type of vegetation.

Scad and Bhar are known to be oligotrophic (Magalhaes, *et al.*, 2016). This is consistent with the pattern seen in this study, with a lack of vegetation observed at both sites. At Scad no stickleback were observed with the ROV and Bhar had the second highest (with Duin). The distribution of stickleback in these two lochs might be determined by the presence of predators, and if the ROV is viewed as a predator it could skew population estimates. Bhar is known to have a low trout population (Spence, *et al.*, 2013), so reactionary avoidance of ROV might be less. No data has been recorded for populations of trout in Scad, although it is known to be one of the prominent trout fishing lochs on North Uist (MacColl, *et al.*, 2013). Using the ROV no stickleback were observed in Scad, however the largest number of stickleback were caught in a single trap at Scad. This suggests that stickleback in Scad could have strong predator-based reactions, in ROV avoidance and in shoaling behaviour. Both Scad and Bhar had CPUEs that were quite different between the two methods, with one method having a high CPUE and the other low. This could be a result of the nature of the loch, with them both having low visibility and little plant life, rather than the method of sampling.

CPUE with the ROV at Hosta was the highest. The time spent in low vegetation combined with the high visibility is likely the reason for the highest CPUE from the ROV. However, comparing just the time spent in low vegetation, Duin had approximately twice the CPUE. Suggesting that either the population density is higher at Duin or that the higher CPUE comes from the presence of anadromous stickleback and the variation in their behaviour to that of residents.

Reactions to the ROV appear to be ecotype specific, with residents being much more skittish than anadromous stickleback. Additionally, foraging behaviour was only observed in anadromous stickleback, and bolting and then hiding was only observed in residents. The response to predators between ecotypes has caused behavioural and morphological differences in stickleback, with the availability of areas of refuge against predators reducing the number of armour plates of an individual (Leinonen, *et al*., 2011). The distribution of stickleback in areas of low vegetation can be assumed to be ecotype specific, as significantly more stickleback were observed in areas of low vegetation at Duin, with most of the stickleback observed being anadromous. Knowing resident stickleback seek cover in vegetation from predators (Leinonen, *et al*., 2011), it can be assumed that the ROV is possibly viewed as a predator by stickleback. It appears that the ROV evokes different predator responses in anadromous and resident stickleback, although it is harder to gauge whether anadromous stickleback view the ROV as a threat or not. Larger stickleback approach a predator more closely than smaller stickleback (Külling & Milinski, 1992) and anadromous stickleback are often more aggressive than resident stickleback (McKinnon, *et al.*, 2012), with higher aggression often meaning an increase in boldness (Huntingford, 1976). Behavioural reactions to ROVs are often species specific and are often greater in smaller species and when an individual is >1m above the substrate (Laidig, *et al.*, 2012). Rabbit fish and North Atlantic codfish actively avoid ROVs (Trenke, *et al.*, 2004), if stickleback similarly actively try to avoid the ROV it could give disproportionate estimates on population densities.

The disturbance of stickleback, and therefore a source of possible population estimate bias, could additionally be affected by the artificial lighting on the ROV. Artificial light has been known to cause reactions in rockfish to move away from the light, although intensity of reaction differed among species (Ryer, *et al.*, 2009). In addition to light affecting fish behaviour, sound is likely to have a similar effect (Popper, 2003), although little is known about this.

3.4.2 Transects

Recording animal densities is often done using transects. Strip transects using an ROV are made difficult by the current-induced drag and the limit of the connecting tether (Ajemian, *et al*., 2015). The main factor effecting the efficiency of ROV in this study was the current-induced drag. At depths between 0.1-0.3m the ROV was greatly affected by the surface currents making the recorded footage shaky and unusable for observations of stickleback. At all depths the current affected the ROV by causing it to drift away from the transect line. To combat movement induced through currents in ROVs deployed from a boat, a clump-weight (a weighted chain link connecting the ROV and boat) can be attached to the tether to help prevent drag, with the addition of being able to maintain a constant depth because of the buoyancy of the drone (Yamamoto, *et al.*, 2009; Haggerty, *et al.*, 2016). The addition of a clump-weight to the ROV used in this study would not be possible due to the shallowness of the lochs surveyed. Studies into shallow water populations using an ROV are limited and none offer an effective solution to alleviate the movement caused by the waters surface.

3.4.3 Minnow traps

There are biases associated with all methods of fish population estimation, with fish behaving differently to differing methods (Fernö & Olsen, 1994). The differences between the different CPUE estimates in this study, especially in low density lochs, suggest that this is also true in the comparison between minnow trapping and ROV estimation. Stochastic differences in CPUE could

be partly due to shoaling behaviour, e.g. if, by chance, no shoals are seen from the drone, but a shoal gets caught in a trap, as apparently happened at Scad. This issue could be a more systematic bias if shoals have a stronger tendency to avoid possible predators (including the ROV). Northern red-belly dace are known to shoal away from a predator (Ashley, *et al.*, 1993), so if similar behaviour occurs in stickleback and if the ROV is observed as a predator, it may cause a systematic bias for population estimates. Trap efficiency significantly decreases for red-belly dace in the presence of vegetation (Dupuch, *et al.*, 2011). When casting off the minnow traps, it is difficult to determine whether the trap will land on open substrate or in vegetation, which could skew the results if there is a bias to traps in a particular area. This would mean that to attain a reliable population estimate, multiple traps would need to be used. Similarly, using the ROV near dense high vegetation resulted in a lower number of observed sticklebacks. Both techniques appear limited in effectiveness of estimating populations in areas of high vegetation. Sticklebacks are more likely to be caught in traps when conspecifics are already inside (Kressler, *et al.*, 2021). The trapping results from Scad support this and are a clear indication of the bimodal distribution that can occur when using minnow traps. This bias also occurs in other capture methods such as gill nets used to catch Arctic char (Finstad & Berg, 2011), but has not been documented in ROVs.

ROV based methods have the possibility of recounting the same individual over a transect, whereas this is impossible with trapping based methods, unless traps are used over successive days which would increase the possibility of recapture unless caught individuals were marked.

3.4.4 Conclusion

In summary, the usefulness of an ROV in estimating population densities of stickleback is roughly equivalent to that of minnow traps. With the highest and lowest CPUE of both methods being from the same lochs. However, the ROV mostly resulted in a higher CPUE than observed with the use of minnow traps,

so the reliability of estimating population density with one method over the other could results in highly varied estimates. There are specific advantages and disadvantages in the use of ROVs over traps. Firstly, setting and collecting traps can take around the same length of time as an hour of transects, however the ROV produces a much higher CPUE in this time than minnow traps, and does not have to be conducted over multiple days. However, CPUE can be increased in minnow traps with the addition of more traps, the ROV on the other hand is limited. Additionally, ROVs allow for a deeper understanding of the habitat being surveyed, that would otherwise only be accessible through diving. ROVs often become hindered in movement by the drift caused by currents and vegetation that can become caught in its propellors and disrupt the line of the transect. Stickleback, especially residents, tend to be found in or near vegetation and reactions cause by the ROV could make individuals bolt and hide within vegetation, possibly skewing population estimates while using the ROV.

To improve reliability of estimates of population density modifications to the ROV to maintain a more constant heading (reduce drag) and maintain a depth just above the substrate (reduce shake from surface water motion) would be advantageous. It would allow for a more accurate estimate of population from a reduced doubt of sightings. Additionally, if ROVs are observed as predators, it could improve CPUE if the ROV was painted to be camouflaged or have a less striking colour.

In conclusion, ROVs are a useful tool for population estimates but, as in most surveying methods, there may be bias in the results that they produce. ROVs would probably produce a better CPUE of anadromous stickleback populations than minnow traps and would probably be a better primary method of research. If population estimates are required of anadromous and resident or just resident stickleback, it would likely be beneficial to use both minnow traps and an ROV in tandem to produce a more reliable estimate.

4. General conclusions

4.1 Sex dependent factors of migration

Migration of anadromous stickleback is sex dependent and is affected by both environmental and behavioural factors. Anadromous females migrate in pulses that have a time-lagged relationship with the lunar phase, with numbers increasing towards the new and full moons, before drastically dropping. The migratory pattern in females suggests that they optimise taking advantage of water and current conditions that favour energy efficiency. This would allow females to prioritise mating over foraging to restore lost energy once they enter breeding areas.

Anadromous males appear not to be influenced by changing conditions in the water from changes in the moon and instead appear to migrate early to increase mating success. On the other hand, females do not appear to be under the same pressure to migrate earlier in the breeding season, but a definitive answer to this would require knowledge of the reproductive success of females spawning at different times during the season.

Significantly more males arrived earlier with these early migrants being smaller than those arriving later. This supports the idea that early migration enhances breeding success, as nest location is known to be a stronger influencing factor than body size in mate selection by females. However, body size still does influence mate selection, which is likely why smaller males migrate earlier.

4.2 Site fidelity

To study anadromous migration, two locations were chosen at opposite sides of North Uist. Any differences in the stickleback that spawn at these sites, would allow for an understanding of whether site fidelity possibly occurs in anadromous stickleback. There was a significant difference in the size of anadromous females, with those from Duin being larger than those at Clachan.

Possibly, anadromous females at Duin are able to take advantage of more productive waters of Loch nam Madadh. Additionally, blackspot was more abundant in Duin, than Clachan, further implying that anadromous stickleback return to the same lochs. Site fidelity in anadromous sticklebacks is an area which requires more research, and is currently mostly speculative.

4.3 Use of ROVs for population density estimates

Population density estimates often are skewed by bias, and it appears that ROVs follow the same limitations. The results from this study demonstrate the bias that both minnow traps and ROVs have in estimating population densities of stickleback. Behavioural traits of freshwater and lagoon resident stickleback and of anadromous stickleback cause each method to produce varying results. The explorative and comparatively bold nature of anadromous stickleback when observed with an ROV meant a higher estimate of population density than in residents. Resident stickleback possibly view the ROV as a potential threat and from observations made in this study are more likely to bolt away and hide. These natures of stickleback ecotypes likely cause discrepancies in stickleback population estimates. Limitations of the ROV as a surveying method additionally arises from the vegetation of the study habitat, with denser taller vegetation yielding poorer estimates of populations than minnow traps. Population estimates are similar in CPUE between the two methods in areas with a higher and lower estimated population.

4.4 Further research

Future research in understanding anadromy in stickleback would benefit from monitoring additional factors. A longer observation period starting in April and ending in August would likely cover an entire breeding period and show if any trends (such as a third female pulse) were missed in this study. Monitoring water flow through a migratory channel would give a better understanding of the relationship between the effect currents have on the numbers of females migrating, and would give more certainty that energy efficiency is a priority in

female migration. Recording the numbers of terns or other piscivorous birds over the observation period, would allow for a better understanding of the risks that could be imposed by earlier migration. Additionally, marking caught individuals would help prevent recapture and minimise skewing of data. Implementing the use of an ROV alongside traps would help give a better estimate of population densities. Both the ROV and minnow traps gave a higher estimate of the proportion of different ecotypes in the presence of both residents and anadromous stickleback, so an estimate based on the two methods would likely give a better estimate of population. Using traps alongside the ROV would also allow for measurements and observations of individual stickleback.

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